ANALYSIS OF THE *COXIELLA BURNETII* TYPE IV SECRETION SYSTEM REGION I DURING INFECTION

By

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ANALYSIS OF THE *COXIELLA BURNETII*

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In Greek mythology, when Odysseus left to go to war with Troy, his older friend, Mentor, was entrusted to the care of Odysseus's palace and his son Telemachus. In modern times, Mentor, has become a term synonymous for an individual that is recognized as possessing qualities of trustworthiness, wisdom, and an individual that is worthy of emulation. To this end, Edward I. Shaw, Ph.D., encompasses all of these qualities and much more. I wish to thank my *mentor* for his guidance, council, and wisdom. He has put up with my youthful ignorance with patience, having the foresight of expectation for whom and what I may become. I thank him for being willing to accept me as his first "experiment" as a graduate student. I also want to thank him for his encouragement and guidance in my life's pursuits beyond Graduate School. I, like Odysseus to his elder friend – Mentor, am proud to call Ed *my friend* as I go forward on my life's battlefield.

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CHAPTER I

LITERATURE REVIEW

COXIELLA BURNETII: BRIEF HISTORY OF DISCOVERY

In 1935, contemporaneous research programs at the Rocky Mountain Laboratory (RML) in Hamilton, Montana; the Laboratory of Microbiology and Pathology in Queensland, Australia; and Queensland Health Department at Brisbane, Australia began the initial investigations into what was thought to be a filterable virus of the *Rickettsiae* family, but was later found to be the Gram-negative bacterium now known as *Coxiella burnetii*.

Public demand around the turn of the 20th century in the mountains of the Bitterroot Valley of western Montana insisted that a cure to the local epidemic of the "spotted fever of the Rockies" led to the establishment of what today is known as the Rocky Mountain Laboratory (RML) [1]. In an effort to study the ecology of Rocky Mountain Spotted Fever (RMSF) and Tularemia, Dr. Ralph R. Parker, one of the founding members of RML [2], collected a sample of *Dermacentor andersoni* ticks from the nearby Saw Tooth Canyon in 1925. From this collection of ticks, Dr. Parker sent a sample to a colleague working at the Rockefeller Institute named Hideyo Noguchi. Dr. Noguchi subsequently isolated what he termed a "filter passing virus," with agent descriptions that are similar to the Nine Mile Isolate (NMI) strain of *C. burnetii* [3]. He subsequently passed the strain in guinea pigs for several generations however later lost the strain during passage. This has prevented the retroactive comparisons of the strain Noguchi isolated with subsequent isolates of *C. burnetii* NMI [2].

In the spring of 1935 RML scientist Gordon Davis, while studying the ecology of RMSF and Tularemia, collected ~200 *D. andersoni* ticks from Nine Mile Creek (Montana) [4]. Dr. Davis divided the ticks into four groups and placed them onto four

separate guinea pigs for a blood meal. Monitoring the guinea pigs for several weeks, he found that two of the pigs remained afebrile, the third died, and the fourth developed a fever on the 12th day post tick feeding [4]. The febrile guinea pig was then used to sequentially infect other guinea pigs, each of which subsequently developed a similar febrile illness [4].

In 1936, Herald R. Cox began working at RML and was assigned by Dr. Parker to work with Dr. Davis on characterizing the Nine Mile agent. Dr.'s Cox and Davis together worked on the NMI strain for several years characterizing numerous facets of the agent including its pleomorphic form resembling a Rickettsia species and that it was "not a filterable virus in the recognized sense of the term" [5]. In his April 1938 monthly report to the NIH director Rolla E. Dyer, Dr. Cox stated that he had discovered how to cultivate the NMI Rickettsiae in embryonated eggs. This report prompted Dr. Dyer to make a personal visit to RML to verify Dr. Cox's claims [2]. Through a series of fortuitous events, Dr. Dyer became infected with a laboratory acquired NMI strain and subsequently linked the pathophysiology of the NMI agent to the Q fever agent simultaneously being worked on in Australia [6].

In 1935, the director general of the Heath Medical Services for Queensland, Australia, Sir Raphael Cilento, approached the Director of the Laboratory of Microbiology and Pathology of the Queensland Health Department at Brisbane – Edward H. Derrick M.D. – and charged him to investigate an outbreak of an unknown febrile illness in Brisbane abattoir workers [7]. Dr. Derrick began his studies into the Query (Q) fever agent by investigating clinical cases of Q fever, as they were manifested. He described a febrile illness lasting from 7 – 24 days, with patient blood and serum samples

being culture negative for common veterinary and zoonotic diseases of the region [7]. He suspected this disease to be caused by a novel agent. This led him to attempt to isolate the agent by infecting guinea pigs with patient blood or urine samples. These methods proved successful in producing the disease in guinea pigs, which he then used to begin a series of animal passages and studies in an attempt to identify the agent. From his investigations, Dr. Derrick mistakenly concluded that the agent was viral and subsequently sent an emulsified saline liver sample to the noted virologist Macfarlane Burnet [7] at the Walter and Eliza Hall Institute [2] in Melbourne, Australia.

Dr. Burnet and his colleague, Mavis Freeman, set out together to further characterize the Q fever agent received from Dr. Derrick. Through subsequent filtration and serum agglutination experiments, they concluded that the agent was a new Rickettsial agent [8]. Dr. Burnet sequentially succeeded in characterizing the unique properties of this organism and its status as a unique species. For this reason, Dr. Derrick proposed the name of *Rickettsia burneti* be applied to the Q fever agent [9].

Around the same time (1939), Dr. Cox proposed a name for the NMI agent to be *Rickettsia diaporica* (diaporica is Greek for having the ability to ‘pass through’, *e.g.*,, a filterable *Rickettsia*) [10]. It was not until 1948 that the classification and nomenclature of the Q fever/NMI agent was finalized. Cornelius B. Philip of RML proposed that the current status of Coxiella (at the time being a subgenus of *Rickettsia*) be elevated to full genus status and that the name be changed from *Rickettsia burneti* to *Coxiella burnetii* [11].

COXIELLA BURNETII: Q FEVER

Q fever has two basic disease manifestations – acute and chronic – which can range from sub-clinical having no recognizable symptoms, to physically debilitating such as endocarditis and in some cases even death [12]. The minimum infectious dose for human acquisition of Q fever typically ranges from 1 to 10 organisms [13]. The typical clinical manifestation of an acute Q fever case is characterized by a febrile illness lasting from 7 – 57 days in untreated individuals [14]. This fever can be accompanied by one or all of the following: a general feeling of malaise, chills, photophobia (light aversion), night sweats, and a general overall feeling of excess fatigue [6]. These symptoms are commonly associated with a host of bacterial and viral diseases, leading to frequent under or misdiagnosis of Q fever cases [15].

Chronic Q fever develops in susceptible individuals after an acute Q fever episode [16, 17]. It has been hypothesized that *C. burnetii* is never entirely cleared from an infected individual following an acute Q fever episode and thus poses a potential source of *C. burnetii* for a chronic Q fever episode in susceptible individuals [16, 17]. Polymerase chain reaction (PCR) analysis has indicated the presence of *C. burnetii* DNA in the bone marrow of convalescent acute Q fever patients independent of chronic Q fever manifestations [16, 17], suggesting the possibility of *C. burnetii* being sequestered in immunologically privileged sites [16, 17]. Until susceptible conditions arise within the convalescent individual, the conditions for Chronic Q fever manifestations remain at bay.

Recently, a 13 year retrospective study evaluated 1,383 confirmed Q fever cases [18]. Of these cases, 77% were found to have had the acute form of Q fever while 23% had the chronic form [18] resulting in a 3.3:1 ratio of acute to chronic cases. Of the

Acute patients, they were classified as with, 40% hepatitis, 20% pneumonia coupled with hepatitis, 17% pneumonia, 17% fever only, 1% meningoencephalitis, myocarditis or pericarditis – respectively, and 0.7% with meningitis only [18], with the remaining 2.3% remaining unclassified. They found that 13 individuals who manifested with myocarditis or meningoencephalitis died [18], a rate of ~1% which is similar to the 1-2% of deaths typically associated with Q fever infections [12]. In this study, age and gender associations with acute Q fever revealed that hepatitis predominated in younger individuals. Pneumonia was manifested mostly in the elderly and in immunocompromised patients, while female patients suffered mostly from isolated febrile symptoms. These data indicate that there are likely many host factors that influence the clinical expression of acute Q fever [18]. Treatment for acute Q fever is usually with tetracycline, rifampin, doxycycline, quinolones, or macrolides for 7 - 10 days [19, 20].

C. burnetii-induced endocarditis is of particular concern following an acute episode of Q fever. After exposure, 40% of people with underlying heart valve defects or prosthetic valves will develop valvular Q fever endocarditis [21]. In many countries, Q fever infection of heart valves is a leading cause of culture negative endocarditis [22, 23]. Treatment for chronic Q fever endocarditis is costly. Antibiotic courses of doxycycline and hydroxychloroquine for 18 - 24 months are often accompanied by surgical removal of the infected valve [24]. Relapse in the repaired valve or other valves of the heart is common [25] making this a particularly insidious chronic infection. It has been suggested that this syndrome is considerably under-diagnosed [22] in culture negative endocarditis cases resulting from *C. burnetii* infections. To prevent the Q fever shift to the chronic state

leading to endocarditis, a combination of doxycycline plus hydroxychloroquine has been shown to be a superior preventative treatment than doxycycline alone [21].

Immunologically privileged and/or poorly accessible immune system sites in the body are the locations where *C. burnetii* safely sequesters itself resulting in chronic Q fever. Some of these locations include:

- Heart valve tissues and the heart lining causing endocarditis and pericarditis [16, 18, 21, 26-32]
- Nervous system and brain resulting in meningitis and encephalitis [18, 33-35]
- Osteoarticular regions causing bone infections [18, 36, 37]
- Hepatic/liver causing hepatitis [38]
- Placenta leading to fetal abortion, low birth weight [18], infantile mortality [39],
- Reproductive organs and tissue leading to possible infertility [40-42]

Chronic Q fever is classically associated with clinical outcomes of endocarditis, hepatitis, and chronic fatigue syndrome [43, 44].

Clinical diagnosis of Q fever is determined by a rise in serological antibody titer to the *C. burnetii* phase II antigen for acute disease and an increase in antibodies to phase I antigen for the chronic form [45-47]. PCR and immunohistochemistry (IHC) have also been used to detect *C. burnetii* DNA and whole cell antigen, respectively, in histological samples [48]. Clinical confirmation of acute Q fever is based on serological evidence from Indirect Fluorescent Antibody (IFA) assays using phase II specific antigens. Acute Q fever antibody titers of IgM $\geq$1:50 and IgG $\geq$1:200 dilutions of patient sera are considered positive [45-47]. For chronic Q fever, antibody titers to the phase I antigen of $\geq$1:800 and $\geq$1:50 for IgG and IgA antibodies, respectively, are confirmatory [45].

COXIELLA BURNETII: VACCINE DEVELOPMENT

Coxiella burnetii vaccination measures have been attempted for years with varied results [12]. Attempts to develop a large-scale, universally effective *C. burnetii* vaccination program has proven complex. A key correlation exists between program design and application with respect to individual vaccine candidates' personal immunological history regarding previous exposure to *C. burnetii* [49]. Individuals previously exposed to the Q fever agent can result in adverse reactions to the vaccination that can range from local to systemic [49-51]. For this reason any attempt to vaccinate individuals must include a prescreening process that detects and excludes individuals with previous exposure to *C. burnetii* [49].

Recently, monovalent phase I vaccines have proven effective against subsequent aerosol challenge models using mice [52], guinea pigs [51, 53] and monkeys [54]. However, research also shows that *C. burnetii* has heterogenic antigenicity [55, 56] so a monovalent vaccine may not prove effective against all antigenic forms of *C. burnetii* [12].

Though the total effectiveness of monovalent vaccines has yet to be fully assessed against heterogenic *C. burnetii* exposures, a commercially available Australian licensed vaccine, Q-Vax, has had favorable results among human volunteers in Australia [54, 57, 58]. Recent work on selection of specific immunodominant antigens and their effectiveness for inducing an immunological reaction providing immunity to *C. burnetii* are in the animal model phase [59, 60]. Should this approach be found efficacious, it may present a safer alternative to the current whole-cell killed vaccines for *C. burnetii*.

COXIELLA BURNETII: EPIDEMIOLOGY

Reservoirs for *C. burnetii* vary widely. *C. burnetii* has been demonstrated to infect livestock and wildlife [61-68], pets [69, 70], birds [68, 71-74], ticks [27, 75], arthropods [12, 76], poikilotherms [68], and amoeba [77]. In the United States, the seroprevalence rates among goat, sheep, and cattle, are 41.6%, 16.5%, and 3.4% respectively [78]. Sheep are the most commonly reported source for human infection [78]. Chronically infected animals shed bacteria in milk and urine [79-81]. Though it does not commonly present itself as an overt disease in goats, sheep, and cattle, *C. burnetii* has a tropism for placental/birthing tissues and causes many spontaneous abortions [82, 83]. Birthing materials (placenta, amniotic fluids) have been shown to harbor on the order of 10^9 organisms per gram [67] that can be aerosolized and infect other animals or people upon parturition.

Domesticated animals present a means by which the animal and human worlds conjoin for *C. burnetii* infections. A particularly interesting demonstration of this involved a pet cat infected with *C. burnetii* delivering kittens in a basement near a group of poker players, resulting in exposure and infection of the players resulting in an urban outbreak of *C. burnetii* pneumonia [84]. A seroprevalence study using PCR and IFA screening methods to detect *C. burnetii* infected domestic and feral cats in Korea and Japan found a significantly higher *C. burnetii* infection rate among feral cats (41.7% versus 14.2% in domesticated cats) [85]. Another study of anti-*C. burnetii* phase II antibody seroprevalence in wild brown rats from various United Kingdom locations found anywhere from 7% to 53% of rats were phase II seropositive [86], suggestive of a potential link between a wild reservoir and domestic animals.

A vast array of domestic and wild birds have been shown to harbor *C. burnetii* including poultry (chicken, turkey, geese, and duck) [67], quail and doves [72], crows [68, 71, 72], sparrows [68, 71], pigeons [68, 71, 73], owls, swallows, and parrots [68]. Birds pose a potential source for zoonotic infection of humans by inhalation of contaminated fomite bird products [12]. In summary, reservoirs for potential zoonotic transmission of the Q fever agent are plentiful in both natural and domestic environments.

COXIELLA BURNETII: GLOBAL DISTRIBUTION

The global distribution of *C. burnetii* has been firmly established [67] with the notable exception of New Zealand [12, 87, 88]. Various searches have been undertaken in New Zealand, but none have found any evidence for the presence of *C. burnetii* [12, 88]. One 1993 study looked at 2,181 bovines and 12,556 canines with recent histories of fetal abortions (a likely indicator of *C. burnetii* infection). Negative results were obtained for the Q fever agent in all animals tested [88].

New Zealand has been given the moniker of the "land of birds." This has reference to the faunal record of the islands. The absence of nonvolant terrestrial (mammalian) fauna prior to the habitation of the original Maori settlers (c1300 A.D.) is well documented [89]. Given the fowl and faunal history of the island country, the conspicuous absence of *C. burnetii* from New Zealand is baffling when one considers two factors, namely migrating fowl returning to the island from distal *C. burnetii* endemic locations, and the importation of livestock (in particular sheep, cattle, and goats) from *C. burnetii* endemic regions such as neighboring Australia. At least one of these factors should have provided an introduction of *C. burnetii* to the island long ago.

Though these routes for *C. burnetii* infection of New Zealand are distinct possibilities, the 1993 study strongly suggests that these have not occurred.

Conversely, the Canary Islands are highly endemic for *C. burnetii*. A recent eleven year study found that among a group of patients with community-acquired pneumonia (CAP), a subset (28%) where found to have a pathogenic source of the disease. Of these 28% CAP cases, it was found that *C. burnetii* was the most common source (20.1%) as compared to other sources such as *Streptococcus pneumonia* (16.6%), *Mycoplasma pneumonia* (15%), *Chlamydophila pneumonia* (13.4%), and *Legionella* sp (8.7%) [90]. Interestingly, Norway remained Q fever free until 1997 when four Norwegian tourists returned from travel to Bhutan, the Canary Islands, and Morocco. Upon reentry to Norway, each presented with acute Q fever [91].

Given the ease and speed of modern travel, it is likely only a matter of time until the first case of Q fever will be found in New Zealand. Once this occurs, all major areas of the globe will have confirmed *C. burnetii* or Q fever cases.

COXIELLA BURNETII: TRANSMISSION

Aerosol transmission of *Coxiella burnetii* is the primary mode of human acquired Q fever. *C. burnetii* aerosols are typically generated as contaminated dust from soils becomes disturbed and airborne [66, 92]. Fomite *C. burnetii* aerosols are generated in zoological associated activities such as animal processing at abattoirs [93, 94] and the birthing of chronically infected livestock which typically exposes veterinarians [69, 95-98] and animal handlers [78] to *Coxiellae*. Actively growing *C. burnetii* in laboratory research settings presents another potential source for aerosolized agent. One case that

highlights the infectivity of *C. burnetii* via aerosolization was reported at a medical school in which pregnant sheep were being used for perinatal research. Unknown to researchers and the animal handling staff, certain of the pregnant sheep were infected and actively shedding virulent *C. burnetii*. When the first cases from this facility were diagnosed and reported, a wider investigation ensued. In total, 137 individuals tested seropositive for phase II *C. burnetii* antigen. Of the seropositive individuals, 41 had been directly involved with the sheep while the remaining 96 were located along sheep carting routes throughout the facility from the animal housing area to the research labs [99].

Ingestion of *C. burnetii* contaminated materials is a second mode of bacterial transmission, though in relation to aerosols this is a far less common. The shedding of *C. burnetii* in milk [55, 63, 100-102] has led to cases of Q fever [103]. *C. burnetii* shed via urine and fecal material [12] and runoff under unsanitary conditions can present a potential fecal-oral infectious route from contaminated water stores. The transmission of *C. burnetii* via fecal-oral and aerosol mechanisms between livestock is likely considering the relative proximity of living conditions in feedlots, stockyards, and barns. It has been speculated that *C. burnetii* infection via ingestion may present a primary mode for hepatic Q fever [12, 103].

Arthropod/Vector borne transmission of the Q fever agent to humans via tick bite is rarely reported in the literature [12]. Recognizing that *C. burnetii* was initially isolated and identified via *D. andersoni* ticks [4], only a select few instances of tick vectored human Q fever cases have been reported in literature [27, 75]. Historically it was thought that *C. burnetii* cycled in ticks via transovarial and transstadial transmission [104, 105] prior to being vectored into humans or animals [105].

Venereal transmission of *C. burnetii* has been demonstrated in animal models [106]. In 2001, a single case was reported of a man who had acquired Q fever via an occupational exposure and subsequently passed it to his wife via intercourse [107]. This method of transmission is rare but possible with individuals shown to have *C. burnetii* in their reproductive tissues as was the case in this report [107].

Cutaneous transmission of *C. burnetii* was suggested in a 1993 report [108]. This report, though not definitive, does present the possibility that a cutaneous infection of *C. burnetii* may be possible; however, it remains an extremely unlikely route for obtaining Q fever.

COXIELLA BURNETII: BIO-WEAPON?

The potential of biological agents for use in bio-weapons development are typically measured against specific criteria. These include the ability to disable troops and/or civilians, potential for mass production, ability to be adapted to a viable delivery system (*e.g.*, aerosolizable), and long term environmental viability [109]. Considering these criteria *C. burnetii* is suitable for development as a biological weapon; however, militarily it is only considered a disabling weapon do to its lack of a high mortality rate as compared to other agents such as *Bacillus anthracis* [13].

The United States and the former Soviet Union are both suspected of having researched and/or weaponized *C. burnetii* [109]. Evidence for its use during armed conflicts is speculative, though several instances of confirmed outbreaks of Q fever amongst soldiers during World War II exits. However, the majority of these outbreaks have been attributed to naturally occurring sources [110-112]. Nevertheless, the United

States government did list *C. burnetii* amongst agents for biological weapons development in 1942 at the biological program at Fort Detrick, MD [109]. In 1954 project "Whitecoat" was carried out by exposing human volunteers to aerosol releases of *C. burnetii* [113].

Recent evidence shows that the cult Aum Shinrinkyo, famous for its March 1995 sarin gas attack on the Tokyo subway system was developing *C. burnetii* for bio-terrorism when it was discovered and disbanded [114]. Various theoretical biological attack scenarios, each modeling *C. burnetii* have been proposed in the literature illustrating the potential threat and possible consequences of a successful Q fever attack [109, 115].

COXIELLA BURNETII: SELECT AGENT STATUS

The United States Select Agent Program fulfills legislative requirements as set apart in the USA PATRIOT Act of 2001 and the Public Health Security and Bioterrorism Preparedness and Response Act of 2002 regarding dangerous biological agents and toxins. The Select Agent Program unconditionally restricts the unauthorized manipulation of designated agents categorized as potential biological weapons candidates [116]. For each candidate agent on the Select Agent list, specific laboratory and personnel approval must be obtained in order to maintain, manipulate, or perform research on the specified agent. This approval comes directly from the designated responsible agency that has been assigned overseer responsibility for each specific Select Agent. All *C. burnetii* strains with the sole exception of *C. burnetii* Nine Mile phase II clone 4 (NMII) {http://www.cdc.gov/od/sap/sap/exclusion.htm} [117] are regulated

under the Select Agent Program, is overseen by the CDC, and are categorized as category "B" Select Agents [118]. *C. burnetii* has been categorized as a category "B" select agent by the United States government because it lacks the potential for massive fatalities that category "A" Select Agents do (*e.g.*, *Bacillus anthracis*, *Francisella tularensis*, and the Smallpox virus) [116, 118].

COXIELLA BURNETII: BIOLOGY

Taxonomic characterization of *C. burnetii* is of the Kingdom: Bacteria, Phylum: Proteobacteria, Class: γ-Proteobacteria, Order: Legionellales, and Family: Coxiellaceae. *C. burnetii* is an obligate intracellular, Gram-negative rod that exhibits pleomorphic characteristics having a distinct poly-phasic life cycle generalized to correspond to stages inside and outside of the host cell [119].

Morphologically distinct forms of *C. burnetii* have been documented in the literature from as early as 1959 when B. Babudieri reported his light microscopy observations of "a very short rod, frequently with a bipolar appearance and sometimes as a minute paired coccus" [67]. Later following electron microscopy observations of *C. burnetii*, he erroneously concluded that "[*C. burnetii*] shows a marked uniformity of dimensions, which shows that the various appearances seen under the optical microscope are due more to differences in dye affinity than to morphologic variations" [67]. The true morphological status of *C. burnetii* was debated until 1981 when transmission electron microscopy (TEM) showed *C. burnetii* as having distinct forms termed large cell variants (LCV), small cell variants (SCV), and spore-like particles (SLP). The approximate sizes for these forms are ~1µm for the LCV with the SCV ranging from 0.2 – 0.5µm and the

SLP varying from 0.13 – 0.17µm [120-122]. Further characterization of the SCV form were made to designate what is called the small dense cell (SDC) form of SCVs. The SDC subset has been shown to resist pressures up to 20,000 lb/in^2 which kills the typical SCVs [123]. Though TEM evidence exists for these SDC and SLP forms, it remains difficult to separate these to purity from the dominant LCV and SCV forms of *C. burnetii*. Considering this limiting fact, the current body of work differentiating the biological forms of SDC and/or SLP is largely hypothetical and has not been tested in-depth; therefore, the role they play in infectivity, disease, and cell survival of *C. burnetii* is unknown [122].

The LCV is considered to be the metabolically active form of the bacterium [120], whereas the SCV and SDC are environmentally stable, metabolically inactive forms of *C. burnetii* [119]. The differences in the LCV and SCV forms of *C. burnetii* can be likened to log-phase and stationary phase growth states of other bacteria, respectively [122]. The different physical states of the LCV and SCV can be characterized by shifts in size, membrane structure, and proteome expression. For example, the major outer membrane protein (MOMP) of *C. burnetii*, designated P1 (~29kDa), is highly expressed in the LCV, down-regulated in the SCV, and absent in the SDC [119]. Studies have shown that peptidoglycan (PG) levels shift as the bacteria changes from SCV to LCV and back to the SCV form. The composition of the peptidoglycan protein complex (PG-PC) of the *C. burnetii* cell wall structure has been shown to be ~2% PG-PC in the LCV, whereas the PG-PC of the SCV is ~32% [124]. It has been hypothesized that partial enzymatic digestion of the SCV PG wall or a possible incomplete synthesis of PG occurs in the LCV to account for this difference in PG-PC content [124].

The differing PG-PC content of the cell wall of the LCV verses the SCV forms, the ultra-structural nucleoid of *C. burnetii* exhibits a marked variation that differentiates these forms. The SCV expresses a histone-like protein designated Hq1 that displays compositional and primary amino acid similarities to the eukaryotic histone H1 homolog [125]. The Hq1 protein is absent (or drastically reduced) in the LCV form of *C. burnetii* [125]. In addition, a small basic protein solely translated in the SCV (designated ScvA), was discovered to have a function analogous to the Hq1 protein [126]. The presence of DNA condensing histone-like proteins in the SCV is logical given its spore-like nature, diminished size, and small electron dense morphology. Recently an in-depth proteomic study of protein/antigen variations expressed in LCVs and SCVs identified a number of form-specific proteins [127]. Using $CsCl_2$ differential centrifugation to separate LCVs from SCVs coupled with 2D gel electrophoresis and mass spectrometry, a series of differentially expressed proteins were identified, 15 in the LCV and 4 in the SCV. Functional roles associated with these expressed proteins were found to be correlate with the metabolically active LCV and structurally stable SCV forms [127].

Both LCV and SCV forms have been shown to be infectious *in vitro* [122, 128]; however, the SCV is widely considered to be the primary infectious form of the bacterium in natural environments as it is likely the form responsible for extracellular survival of the bacterium in the environment [122]. The LCV is considered to be the metabolically active and primary host cell-to-cell spreading of the infectious form of *C. burnetii in vitro* and presumably *in vivo*, although it is environmentally unstable [122]. Recently the cell cycle kinetics of *C. burnetii* infection *in vitro* were thoroughly investigated [129]. Using SCVs to initiate infection in Vero cells it was demonstrated

that defined lag, log and stationary phases of *C. burnetii* growth exist [127, 129]. The SCV form of the bacterium predominates during the first 2 days post infection (PI) as SCV to LCV morphogenesis occurred (analogous to a lag phase). From day 2 to day 6 PI, the LCV form was found exclusively within infected cells and exponential replication occurred (exponential phase). After day 6 PI, both LCVs and SCVs were shown to exist in cells as LCV to SCV reversion began to occur (stationary phase) [127, 129]. Extracellular signals presumably effect this biphasic conversion of *C. burnetii* forms, yet the cellular signals and bacterial mechanisms involved remain relatively unknown [122].

SCV and LCV forms of *C. burnetii* each play pivotal roles in the infectious life cycle. Conversion from SCV to LCV and back to SCV forms occurs, allowing *C. burnetii* to maintain its ability to be environmentally stabile in the SCV form, while intracellular conversion to the LCV provides for its metabolic necessities and replication. It is then shed into the environment as the SCV form where it will reside until it is passed to a new host and reinitiates its infectious cycle [122].

COXIELLA BURNETII: ENVIRONMENTAL STABILITY

Environmental stability of *C. burnetii* SCV has been demonstrated under various chemical and radiation sterilization methods. Chemical and physical treatments of *C. burnetii* using methods which sterilize most bacteria (including *Bacillus* spores) show little effect on *Coxiellae* [130, 131]. Additionally *C. burnetii* shows resistance to UV, desiccation, osmotic shock, sonication (in distilled H_2O >30 min.) and elevated temperatures (63°C for 30 min.) [122, 130]. Following 24 h treatments with 0.5% sodium hypochlorite, 2% Roccal, 5% Lysol, or 5% formalin infectious *C. burnetii* were

still detectable while *Bacillus* spores were inactivated under the same conditions [130]. However, 70% ethanol, 5% chloroform, or 5% Enviro-Chem have been shown to be effective in inactivating *C. burnetii* with 30 min of treatment [130]. These results have led researchers to speculate that the environmental stability of *C. burnetii* is related to the "sporulation process" which produces the SCV and SDC forms [130].

The minimum dose of gamma irradiation required to inactivate 90% of *C. burnetii* Phase I and II under stabilizing conditions (-79°C in blended yolk sack membranes) has also been determined. Depending on the phase of *C. burnetii*, the 90% inactivation values are 0.64 and 1.2 kGy (kilogray units) for Phase I and Phase II, respectively [131]. In all samples, viable *C. burnetii* were found following a 5 kGy dose; however, following a 10 kGy dose no retrievable viable *C. burnetii* was found [131]. Of interest, it was found that gamma irradiation up to 20 kGy had no demonstrated effect on cell-wall morphology or surface antigenic epitopes of the cells as compared to non-gamma irradiated *C. burnetii* cells [131].

INTRACELLULAR GENETICS

Genetic manipulation of bacteria is a cornerstone of modern molecular pathogenesis research; however, developing genetic systems for the obligate intracellular bacteria has remained particularly difficult. Foreign DNA can be introduced into *C. burnetii* by electroporation [132-134], yet propagation of clonal isolates has, until recently, proved elusive. Only recently it was reported in the literature that a stable isolate of a *C. burnetii* *fts*Z (transposon disrupted) mutant was isolated. The genetic manipulation was accomplished using a *mariner*-based *Himar1* transposon mutagenesis plasmid containing

the mCherry fluorescence protein with a chloramphenicol antibiotic resistance gene [134]. As the ability to site specifically mutate the *C. burnetii* genome develops, it should led to an accelerated understanding of critical components of the genetic systems of *C. burnetii*.

The *Coxiella burnetii* Genome: Pulse field gel electrophoresis restriction fragment studies estimated the full-length *C. burnetii* genome size to be between 1.6 – 2.1 x 10^6 base pairs [135, 136]. The complete genome sequence of the *C. burnetii* NMI RSA493 isolate was published in 2003 [137]. The genome sequence revealed its size to be 1,995,275 base pairs with a 42.6% GC content. The genome is predicted to encode 2,094 open reading frames (ORFs), of which 1,022 show homology to known genes [137]. Approximately 45% of the predicted encoded proteins have a pI greater than 9.0 making a large portion of *C. burnetii* protein's content very basic. The basic nature of these proteins has been hypothesized to function as a "proton sink," buffering *C. burnetii* from excess H^+ ions found in the phagolysosome environment [137].

The genome sequence also revealed 83 pseudogenes and 32 insertion sequence (IS) elements, 21 of these being copies of the same IS1111 element [137]. Sequence analysis also revealed *C. burnetii* genes associated with adhesion and invasion (13 ankyrin repeat-containing proteins), intracellular trafficking, detoxification mechanisms (multi-drug efflux pumps), and host-cell modulation mechanisms (Type I, II, and IVB secretions systems) [137]. The lifestyle of *C. burnetii* highly resembles Rickettsiae and Chlamydiae bacteria; however, the genome sequence is decidedly different from these other intracellular bacteria species. In fact, the genome content indicates that the obligate intracellular lifestyle of *C. burnetii* is likely a recent adaptation [137].

Antibiotic Resistance in bacterial pathogens, particularly a bio-threat organism like *C. burnetii*, is concerning. The current antibiotic groups used for treating acute and chronic forms of Q fever include the tetracyclines, rifamycins, quinolones and macrolides [19]. However, studies show varying efficacy effects with these antibiotic groups [138]. Although, some data indicate the presence of doxycycline resistant strains emerging in humans and animals [139]. Evidence exists that suggests the ability of individual *C. burnetii* strains to produce acute vs. chronic disease may have a direct correlation to the bacterium's antibiotic resistance capabilities [140]. The genome sequence of the NMI isolate revealed that it contains a high proportion of multi-drug efflux pumps (MDEP) per mega-base of genome sequence (~11 MDEP/Mbase). More than 25% of the transporters encoded in the NMI genome are predicted to be MDEPs. The high proportion of MDEPs encoded by the NMI genome exceeds those of all other known Proteobacteria bacteria [137]. It is speculated that this high proportion of MDEPs in the NMI genome may be related to the harsh environment of the phagolysosomal environment that *C. burnetii* inhabits, and that these MDEPs are used to shuttle host cell defensive measures (defensins, etc) out of the bacterial cell [137].

Rickettsiella grylli: Taxonomic and phylogenetic studies of *Rickettsiella grylli* have shown it to be the closest relative of *C. burnetii* [141, 142]. *R. grylli* is an intracellular parasite of arthropods such as grasshoppers [141] and crickets [142]. It has a bi-phasic life cycle akin to the Chlamydial reticulate and elementary body forms [143, 144]. Murine infection studies using *R. grylli* demonstrated a lack of cytological, serological, or electron microscopic effects [145]. Evidence shows that *R. grylli* genetically diverged from *C. burnetii* approximately 350 million years ago [146].

Genetically the genome for *Rickettsiella grylli* has been shown to be ~2.1 mega base in size [147] roughly 1kb larger than the *C. burnetii* genome. Efforts are underway to sequence the full genome of *R. grylli* to look for possible clues into core genetic features common between *R. grylli* and *C. burnetii* [141].

Legionella pneumophila: Morphological distinctions of *C. burnetii* and its "Rickettsial-like" nature have historically categorized *C. burnetii* amongst the *Rickettsial* species of bacteria [137]. 16S rRNA gene sequencing analysis [148] coupled with protein and phylogenetic analysis of conserved proteins [137] demonstrated *C. burnetii* to be closely related to *Legionella pneumophila*. Subsequently, *C. burnetii* was re-categorized as a member of the Proteobacteria, order Legionellales. *L. pneumophila* is a facultatively intracellular bacterium that exhibits a life cycle akin to, yet distinct from that of *C. burnetii*. *L. pneumophila* lives inside a specialized phagosome yet it actively prevents lysosomal fusion [149] whereas *C. burnetii* requires lysosomal fusion to occur to develop a mature phagolysosome. The published genome sequence of *L. pneumophila* indicated a total genome size of ~3.4 Mbp. Surprisingly, ~42% of the *L. pneumophila* genes have homology to the *C. burnetii* genome despite *Coxiella*'s reduced genome size of ~1.9 Mbp [150].

Coxiella burnetii plasmids: Four plasmids have been found associated with *C. burnetii*. *C. burnetii* isolates contain one of four basic plasmid types; QpH1, QpRS, QpDV, or QpDG [151] at a relatively low copy number (1-5) [152]. The QpH1 plasmid found in the NMI strain is comprised of 37,393 base pairs that encode 40 predicted ORF's [152]. It has been proposed that plasmid type plays a role in whether *C. burnetii* infections cause

either an acute or chronic disease [153]; however, this remains a debate within the field [154, 155].

COXIELLA BURNETII: PHASE VARIATION

The phase variants of *C. burnetii* LPS have been likened to the classic smooth vs. rough LPS of enteric gram negative bacteria, with phase II LPS being equivalent to the rough form while phase I is equivalent to the smooth virulent form [156]. With continued *in vitro* passage of phase I *C. burnetii*, it has been demonstrated that overtime the virulent phase I LPS shifts to a truncated, avirulent phase II form [156].

A study on the DNA of phase I *C. burnetii* Nine Mile Clone 7 (NMI) and phase II *C. burnetii* Nine Mile Clone 4 (NMII) strains using restriction endonuclease digestion concluded that the isolates differed genetically (as clonal isolates) but did not in show antigenic phase variation [157]. Results from restriction fragment length polymorphism studies using *Hae*III digestion of genomic DNA from NMI and NMII strains showed the absence of one *Hae*III fragment from NMII in the comparative patterns. Additionally, a comparison of the NMII strain (which does not survive animal passage) to the *C. burnetii* Grita M44 phase II strain (which is capable of passage in animal models) showed the presence of the missing *Hae*III fragment in the Grita M44 phase II strain. These data suggest that the *Hae*III fragment contains information needed to allow survival of the NMII *in vivo* [158].

Comparisons of cosmid clones derived from *C. burnetii* NMI and *C. burnetii* NMII strains have shown an 18 kb deletion in the genome of *C. burnetii* NMII [159]. In the same study, an additional comparison was made between the intermediately virulent

C. burnetii Nine Mile RSA514 Crazy strain (Crazy Q), non-virulent NMII, and the virulent NMI. This showed a 29 kb deletion in Crazy Q that overlapped the 18 kb fragment also deleted in NMII strain. The deletion of both strains showed had a common terminus. This indicated that the Crazy Q included an additional 11 kb deletion immediately upstream of the common 18kb deletion for these strains [159]. This remains interesting as Crazy Q remains moderately virulent, while NMII does not, despite the fact that they each miss the same 18 kb fragment. This may indicate some importance for the additional 11kb deletion in the Crazy Q strain verses the common 18kb deletion.

The lipopolysaccharide (LPS) molecule of the *C. burnetii* gram negative cell wall has been shown to be a virulence factor [155] directly associated with the severity of Q fever disease [160]. Three LPS structural variants have been identified in different *C. burnetii* phase variants [161, 162]. The three LPS forms have been shown to correspond to the antigenicity of their respective phase variant [160]. *C. burnetii* with phase I LPS is highly infectious causing seroconversion and Q fever in the host with bacterial recovery from the host cell 30 days PI [160]. Although phase II *C. burnetii* strains are 10 times more infectious than phase I strains in cell culture models [160], phase II *C. burnetii* lacks the ability to cause Q fever associated symptoms in animal models and cannot be recovered from animals at 30 days PI [160]. This same study tested the virulence from the Crazy Q strain of *C. burnetii*. Which showed high infectivity with moderate Q fever symptoms in animal hosts, but it was not recoverable 30 days PI [160].

Recent size exclusion chromatography experiments on phase I LPS have shown that the O-polysaccharide affects the antigenic reactivity. Methylation linkage analysis demonstrated the presence of terminal virenose, dihydrohydroxystreptose, and mannose

sugars. Serological evidence implicates virenose and dihydrohydroxystreptose as key glycan antigens involved in the immunobiology of Q fever pathogenesis [163]. The lipid A portion of the LPS of both phase I and phase II has been shown to be identical [164].

The avirulent phase II LPS has been shown to have a genomic deletion linked to the previously described 18 kb deletion [159]. The O-antigen biosynthesis gene cluster was shown to be contained within this 18 kb deleted span [165]. The absence of the O-antigen gene cluster presumably results in the attenuated phenotype of the phase II LPS molecule [160, 165]. This common 18 kb deletion of Crazy Q [159] suggests a similar phenotype; however, Crazy Q maintains a medium length LPS and remains moderately virulent *in vivo*.

The virulence differences between the three LPS variants relate to the shielding effect provided by full-length *C. burnetii* LPS as opposed to truncated LPS forms [166]. Studies using *C. burnetii* NMI Phase I verses *C. burnetii* NMII Phase II treated human dendritic cells (DC) demonstrated that the NMI strain (having full length LPS) prevented DC maturation, allowing *C. burnetii* NMI infections to persist. However, DC infections with the NMII strain resulted in DC toll-like receptor (TLR) 4-independent maturation with production of interleukin-12 and tumor necrosis factor. Additionally it was shown that NMI LPS did not fundamentally alter the ability of the DC cells to mature as subsequent application using purified *E. coli* LPS, or the combination of a super-infection with NMI infected DCs subsequently infected with NMII, resulted in DC maturation in each instance. Subsequent tests using NMI LPS-minus cells were shown to be infectious in DCs; however, these cells were unable to prevent DC maturation [166]. These results suggest that full-length *C. burnetii* NMI Phase I LPS functions to mask DC TLR ligands

preventing maturation thereby allowing the *C. burnetii* bacterium to persist within the DC [166].

COXIELLA BURNETII INTRACELLULAR PATHWAY

Obligate intracellular bacteria occupy a unique niche in the microbial world. Their survival and replication strategies could be compared, in a rudimentary sense, to that of viruses. Like viruses, intracellular bacteria establish themselves inside and replicate within a host cell employing a parasitic strategy in the attempt to circumvent the host's immune system by existing in a protected intracellular environment [167]. Some bacteria that have been shown to inhabit intracellular niches include, *Rickettsiae, Chlamydiae, Ehrlichiae, Legionellae* and *Coxiellae* species of bacteria. Each of these intracellular bacteria utilizes an alternative intracellular strategy for replication.

Rickettsiae species exhibit an "in and out" strategy of intracellular parasitism as they enter the cell via endocytosis and immediately leave the endocytic vesicle environment to replicate in the cytoplasm [168]. *Chlamydiae, Ehrlichiae,* and *Legionellae* are found in *early endosomal*, *mid to late endosomal*, and *late endosomal* intracellular sites, respectively. They enter cells via endocytosis and then subvert the endosomal pathway where they replicate in these unique environments [168]. Only the *C. burnetii* resides in a compartment which appears to resemble a completely mature phagolysosome [168, 169]. It is in this harshest of intracellular environments that *C. burnetii* survives and replicates. During a naturally acquired infection (via aerosol), *C. burnetii* initially targeted by alveolar macrophages upon entry to the host lung. Once inside the macrophage, *C. burnetii* can disseminate throughout the host and infect a variety of host

tissues [122]. Once *C. burnetii* has been shuttled into the acidified phagolysosomal vacuole of the host cell [155], the *C. burnetii* containing compartment is referred to as a parasitophorous vacuole (PV). It is here that the organism lives and replicates with a 10 – 12 hour doubling time in exponential phase [129, 170].

The *C. burnetii* uptake/internalization process by the host cell occurs via a microfilament dependent endocytosis mechanism [171]. In human monocyte THP-1 cells it has been shown that attachment of *C. burnetii* NMI induces protein tyrosine kinase activities that result in phosphorylation of tyrosine residues on host proteins including Src-related kinases, Hck and Lyn, that co-localize to the F-actin cytoskeleton. This activity results in the reorganization of F-actin [172] causing pronounced membrane protrusions at theTHP-1/NMI interface within 5 minutes of interaction which are no longer visible by 60 minutes PI [173]. Conversely, this activity does not occur with the avirulent NMII strain despite its ability to be more efficiently endocytosed [173]. This may be attributed to the truncated LPS of NMII making the bacterium highly hydrophobic [174] which may increase the probability that non-specific hydrophobic interactions between pathogen and host cell, thus increasing NMII's likelihood for uptake [171]. Pretreatment of L cells with proteases (pronase, subtilisin or subtilopeptidase A) prior to infection with *C. burnetii* drastically inhibits bacterial attachment reducing cellular uptake, suggesting the involvement of a host cell surface exposed protein in the *C. burnetii* endocytic process [171].

Possible host binding domains include complement receptor 3 (CR3) and TLR4. Uptake of *C. burnetii* was investigated using a series of CR3 knockout experiments in THP-1 cells [175]. These experiments indicated a need for CR3 on pseudopodal

extensions for *C. burnetii* uptake to occur. Another study indicated that the uptake of virulent Phase I *C. burnetii* is dependent on TLR4 in macrophages through an interaction with the LPS. This activity was not seen with Phase II variants of *C. burnetii* [176]. Of particular interest in this study was that they were able to demonstrate that TLR4 had a role in the induction of F-actin rearrangement in macrophages; however, the TLR4 did not have any effect on phagosomal maturation or macrophage microbicidal activity on *C. burnetii* as TLR4 (-/-) mutants showed no deviation from the wild-type in the intracellular fate of the bacterium once uptake had occurred [176]. Even in knockouts of the possible host ligands, significant numbers of cells become infected [175, 176] indicating that multiple routes of entry are likely used by the pathogen.

Though the story of *C. burnetii* uptake is beginning to be understood, there is currently little data regarding the specific bacterial and host cell receptor mediated ligand(s) required for *C. burnetii* uptake. Given the variety of hosts *C. burnetii* is capable of infecting, it is unlikely that the organism requires a host specific mechanism to mediated cellular invasion. It is likely that *C. burnetii* passively allows the host to internalize it by phagocytosis. Once *C. burnetii* is internalized into a phagosome, it begins to manipulate this compartment. The host cell protease treatment experiment previously described do seem to suggest at least some role for *C. burnetii* interaction with a host cell surface protein; however, it remains to be determined whether this is an active or passive interaction between ligand(s).

Upon internalization, the *C. burnetii* containing vacuole begins to follow the standard endocytic pathway to lysosomal fusion [169], eventually acquiring the attributes of a secondary lysosome [122]; however, the process appears to be delayed for

approximately two hours [177]. Recent publications indicate that during this two hour delay, the *C. burnetii* [178] PV interacts with the autophagy pathway, the PV membrane becoming decorated with the autophagosomal marker LC3 as early as five minutes PI [179, 180]. The autophagy data are beginning to shed light on some of the specific activities occurring during this two hour delay [127]. The mature PV membrane eventually co-localizes with cathepsin D (peptidase) [181], lysosomal-associated membrane glycoproteins 1 and 2 (LAMP1 and LAMP2) [169], 5'-nucleotidase (nucleic acid hydrolytic enzyme) [182], Rab7 (small GTPase and late endosomal marker) [180], and acid phosphatase (lysosomal phosphatase) [183]. Once the PV has fused with the lysosome the PV becomes acidified to a pH of ~4.8 [184].

pH levels within the PV appear to play a vital role in the metabolic processes of *C. burnetii*. The proton motive force generated as the PV acidifies creates an electron gradient across the membrane of *C. burnetii* that is harvested as fuel for its metabolic activities [185]. An *in vitro* cell free model system has been used to investigate *C. burnetii*'s metabolic activity [168, 184]. When comparing the metabolism of glucose and glutamate using the cell-free acid activation model, *C. burnetii* metabolism was highest at a pH of ~4.7-4.8 as compared to neutral (7.0 pH) or slightly alkaline pHs. In fact, neutral or slightly alkaline pHs actually abolish *C. burnetii* metabolism [184]. These results have led to speculation that the conservation of metabolic energy at neutral or slightly alkaline pH contributes to extracellular survival of the organism [185, 186] in the SCV form.

Once *C. burnetii* enters the host cell it is clear that it begins to have an effect on the fate of the endosomal compartment. As early as five minutes post entry into the host cell *C. burnetii* guides the fate of its endosomal compartment by directing it to interact

with the autophagy pathway [179, 180]. Recently two studies demonstrated the essential role of *de novo* protein synthesis that affects maturation of the PV and its enlargement. Using bacteriostatic concentrations of either chloramphenicol (protein synthesis inhibitor) or carbenicillin (cell wall synthesis inhibitor), it was shown that active metabolic activity was required for proper *C. burnetii* trafficking within the host [187]. The chloramphenicol treated cells contained a series of individual tightly bound small LAMP-1 positive PVs each containing single *C. burnetii* cells dispersed throughout the host cell cytoplasm [187]. Conversely, it was shown within the carbenicillin treated cells that mature large spacious PVs formed with multiple non-replicating *C. burnetii* therein [187]. Subsequent removal of chloramphenicol treatment showed vacuoles fused forming large spacious PV's with multiple *C. burnetii* cells [188]. Additional chloramphenicol experiments using *C. burnetii* infected cells combined with the addition of latex beads were performed showing subsequent to chloramphenicol removal that the *C. burnetii* PVs fused with PVs containing latex beads. Suggesting that active *C. burnetii* specific protein synthesis was critical for PV fusion within the host cell [188]. This has led to the idea that directed vesicular recruitment by *C. burnetii* is a primary means whereby *Coxiellae* obtain metabolites and nutrients as precursors for cellular metabolism [122]. Though the interactions involved in the autophagy and PV maturation/recruitment processes remain unknown at present, it is very likely that *C. burnetii* is directing the fate of its environment from inside the PV by effector mediated mechanisms.

SECRETION SYSTEMS

Bacteria live and interact with their varied environments via a host of diverse systems that are essential for their survival including, quorum sensing and conjugation systems, antibiotic and toxin release mechanisms, as well as motility and chemotaxis mechanisms. These systems involve precise sensory response machinery that allows bacteria to detect and react to external and internal environmental signals. Of particular interest are bacterial secretion systems used to manipulate bacterial surroundings by emitting effector molecules via secretary machinery. Secretion systems such as the type I and type II secretion systems have been shown to be relatively universal in bacteria and have been shown to perform a variety of functions related to cellular housekeeping activities [189, 190], while virulence associated functions have been attributed to the type III secretion system [191, 192].

Bacterial conjugation is a method used to shuttle genetic material among neighboring populations of bacteria. Work has shown a variety of plant and animal pathogens using evolutionarily related conjugation systems as a means to deliver various effector molecules [193]. All ancestrally related conjugation machines used to mediate DNA or protein translocation are categorized as Type IV Secretion Systems (T4SS) [194]. As such, this definition is inclusive for all Gram-negative and Gram-positive bacterial DNA translocation machinery with genetic homology to conjugal machinery [195]. The T4SS is similar to the T3SS in that pathogens with a variety of lifestyles appear to possess it [196-198] and may deliver conjugated nucleoprotein and/or protein substrates to neighboring cells. The T4SS was originally found in *Agrobacterium tumefaciens*, *Bordetella pertussis*, *Helicobacter pylori* and *Legionella pneumophila* [193]. T4SS

possessing bacteria have a wide and varied range of target cells including animal, plant, fungal, and bacterial cells [194].

Two basic T4SS have been characterized: the *Agrobacterium* VirB/D4 and the *Legionella* Dot/Icm (*a.k.a.* Icm/Dot) secretion systems. Given the loose definition of the T4SS, a distinction between the differing ultra-structural T4SS types was necessary as subsequent organisms were found to possess varying T4SS [199]. Designated as A and B, relative to the *Agrobacterium* VirB/D4 and *Legionella* Dot/Icm systems respectively [200].

The VirB/D4 T4SS is encoded by two operons, *virB* and *virD* having 11 and 5 genes respectively [195]. The *virB* operon codes for the cell envelope spanning and extracellular filament extrusion structure of the type four A secretion system (T4ASS) structural apparatus, whereas the *virD* genes code proteins associated with T-DNA transfer via the T4ASS [195].

The *L. pneumophila* Dot/Icm type IV B secretion system (T4BSS) includes a series of 26 genes coded on two separate pathogenicity islands in the genome called region I (RI) and region II (RII) [199], each of which consist of *dot* and/or *icm* genes. Fourteen of these genes show homology to the conjugal transfer apparatus of the *tra*/*trb* genes of the R64 and col1b-P9 self-transmissible IncI class conjugation plasmids [199, 201]. Though the homology of the T4BSS genes demonstrate relatedness to these plasmid transfer systems, evidence indicates that the T4BSS machine has been modified to transport effector proteins to the host cell [199].

Investigations into the intracellular growth requirements of *L. pneumophila* T4BSS gene mutants revealed ***d***efective in ***o***rganelle ***t***rafficking (*dot*) or ***i***ntra***c***ellular

multiplication (*icm*) genes required for intracellular growth in macrophages [202, 203]. Most of the T4BSS *dot/icm* genes (25/26) exist within either region I or II with the only exception being DotV which resides exterior to these regions [199].

The physical structure of the *L. pneumophila* T4BSS is currently being characterized. Using a variety of genetic and biochemical analyses, a *L. pneumophila* T4BSS core transmembrane spanning structure demonstrated functional conservation with the distantly related Agrobacterium VirB7-10 T4ASS subcomplex [204]. In *L. pneumophila* the core structure is composed of a dimer pair of DotF and DotG proteins that extends from the inner to the outer membrane where they independently associate with DotH/DotC/DotD proteins [204]. This well designed core transmembrane model of the *L. pneumophila* T4BSS may prove similar for the *C. burnetii* T4BSS.

The location of the remaining proteins of the *L. pneumophila* T4BSS was determined using a combination of differential centrifugation and western blot analysis to identify protein associations with the outer membrane, periplasm, cytoplasm and/or inner membrane of the bacterium. The outer membrane associated protein DotK has a transient association with DotC and DotD prior to these (DotC and DotD) associating with the core transmembrane structure (DotF, DotG, and DotH) on the inner face of the outer membrane [204]. The periplasm contains IcmX and DotH, with DotH then moving on to its association with the core structure on the outer membrane face [204]. The IcmQ, IcmR, DotB, IcmS, and IcmW proteins were all located in the cytoplasm [204]. The inner membrane shows the greatest number of T4BSS associated proteins and includes DotL, DotM, DotN, DotO, DotP, DotI, DotA, DotE, DotV, DotJ, DotU, IcmF, IcmT, and

IcmV with DotF and DotG interacting initially with the inner membrane prior to their association with the core complex of the T4BSS [204].

Various studies on the function of individual *L. pneumophila* T4BSS proteins have been performed identifying protein specific activities of the *L. pneumophila* T4BSS. Substrate adapters were identified in DotU and IcmF that act to stabilize DotH as *L. pneumophila* transitions to its virulent stationary phase [205]. Additionally, a homo-hexameric ring of DotB proteins was identified that acts as a functional ATPase, likely providing the energy necessary to drive the T4BSS machinery [206]. It was also noted that DotB has significant homology to the PilT family of proteins known to mediate pilus retraction, which may be indicative of an additional function for the DotB protein [206, 207]. IcmS and IcmW appear to form a complex to mediate substrate/protein interactions for translocation via the T4BSS into the host cell [208, 209]. A bacterial two-hybrid screen using DotF as bait found that it interacts with a variety of secreted substrates [210]. IcmR was found to bind to the N-terminal region of IcmQ in a chaperone/substrate relationship and to act as a regulatory protein for IcmQ function [211, 212]. IcmQ was also shown to insert into lipid membranes and form pores which allow the dye calcein, but not Dextran 3000, to efflux the lipid membrane. This suggests a pore size restriction for effector substrates via the IcmQ pore [212]. The DotF data supports a model where DotF form an integral component of the transmembrane structure for the core T4BSS, and requires DotF to be capable of interacting with all T4BSS substrates. The IcmQ data also fit this model in that IcmQ may serve as an inner membrane pore allowing certain substrates to enter into the periplasmic space for subsequent type IVB secretion.

The *C. burnetii* genome sequence revealed both type I and II secretion systems and two loci within a 33kb span of DNA containing ORFs that have a high degree of homology to the T4BSS genes of *L. pneumophila* [137, 199]. Of the 26 *L. pneumophila* T4BSS genes, homologs of 23 appear to be encoded in the *C. burnetii* genome [213]. Of these 23 genes of the *C. burnetii* T4BSS, 22 are encoded within the two gene/linkage groups (designated Region I and II, respectively) of the 33kb span. The sequence homology and genetic organization of the *C. burnetii* and *L. pneumophila* T4BSS genes led to the assumption that *C. burnetii* has a functional T4BSS which is used to secrete effector proteins into its host cell.

To determine whether the putative *C. burnetii* T4BSS proteins are functionally homologs, complementation studies expressing cloned *C. burnetii* genes in *L. pneumophila* T4BSS mutants were performed. Combined, these studies indicate that the *C. burnetii* T4BSS genes *icmS, icmW, dotB,* and *icmT* (in one model) could complement the corresponding *L. pneumophila* mutants, while *icmQ, icmP, icmO, icmJ, icmB,* and *icmX* could not [214, 215]. Another study analyzed total RNA from Vero cells infected with avirulent *C. burnetii* NMII using RT-PCR and found *icmS, icmW, icmQ,* and *dotB* were expressed by 48 hours PI, with *icmW* detected as early as 24 hours PI [214]. While the *C. burnetii* T4BSS is strikingly similar to the *L. pneumophila* T4BSS, differences do exist that preclude complementation with all homologous proteins. Considering the differing life-cycles and environments in which they replicate, this is not surprising.

Figure 1.1 shows a physical map of the genetic loci for the *L. pneumophila* and *C. burnetii* T4BSS. Region's I and II show a high similarity in composition and orientation of genes between the two systems. Region I of *L. pneumophila* shows a series of non-T4BSS

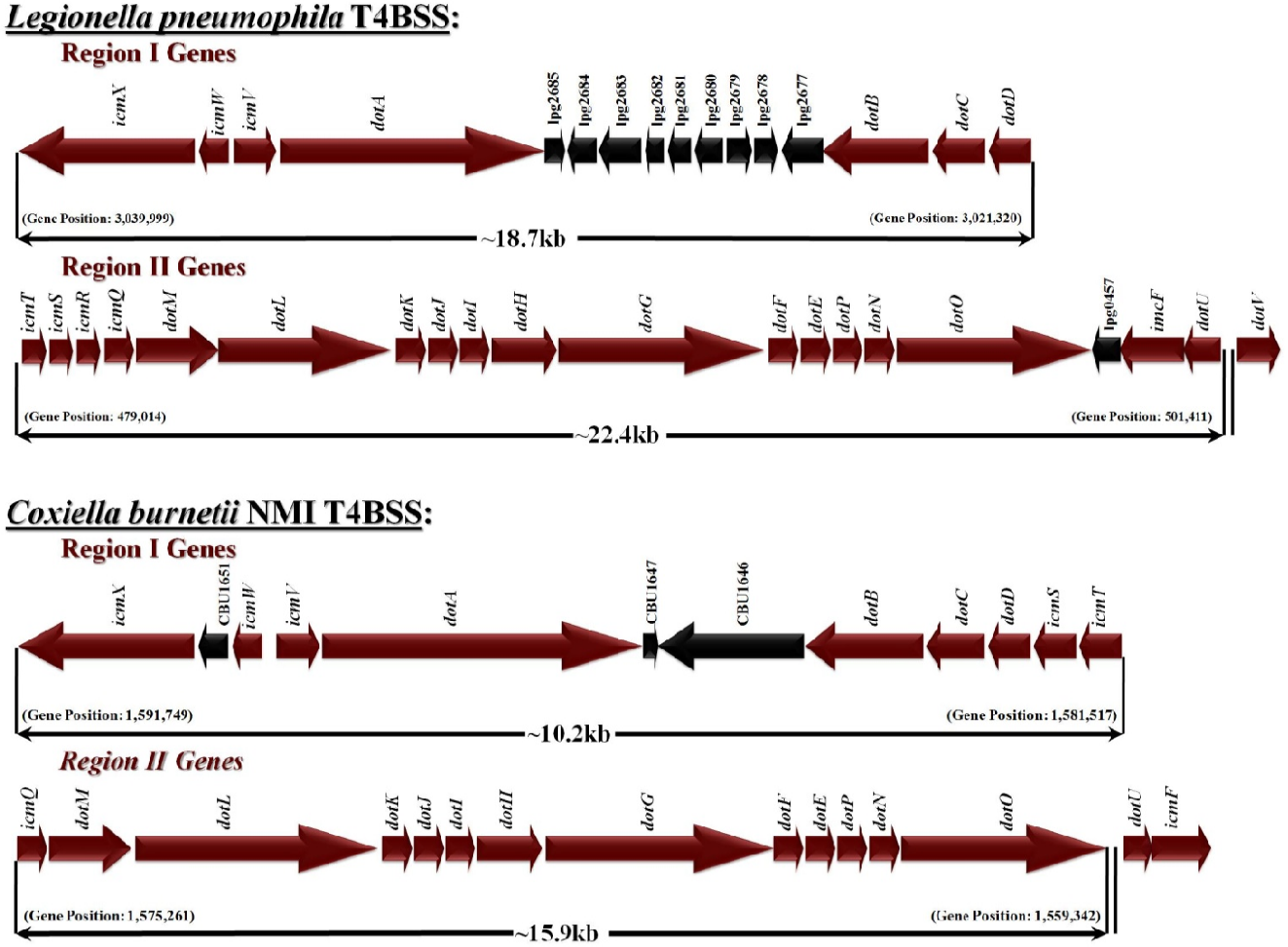

Figure 1.1: A comparative physical map of the *Legionella pneumophila* and *Coxiella burnetii* Dot/Icm secretion system loci. Black genes are non-TFBSS genes.

genes that separate the convergent *dotA* and *dotB* genes, while in the *C. burnetii* region I a single non-T4BSS gene exists between *icmW* and *icmX*. Region II of *L. pneumophila* shows one gene (lpg0457) which separates the convergent T4BSS genes *icm*F and *dot*O, while the *C. burnetii* region II has no non-T4BSS genes and ends with *dotO*; the *C. burnetii dotU* and *icmF* lie outside of the region. The *L. pneumophila* T4BSS *icmR*, *dotJ*/*icmM* and *dotV* genes apparently do not have equivalent genetic homologs in the *C. burnetii* T4BSS [213]. The genome sequence information showing T4BSS homologs in *C. burnetii* extends previous work that had identified randomly sequenced *C. burnetii* fragments with homology to the *L. pneumophila* T4BSS proteins [216].

Though not extensively characterized, the regulation of RNA expression within *L. pneumophila* Regions I and II was analyzed by constructing nine *Icm*::*lac*Z fusions [217]. Site-directed and PCR random mutagenesis of these fusions identified DNA regulatory elements. The *icmT*, *icmP*, *icmQ*, *icmM*, *icmV*, *icmW*, and *icmR* genes were all found to contain a 6-bp sequence (TATACT) essential for their expression. Primer extension showed this sequence serves as their -10 promoter element. In addition, sequences upstream of *icmV*, *icmW*, and *icmR* contained regulatory elements appearing to serve as binding sites for transcriptional regulators. Altogether, twelve regulatory elements, seven of which constitute the -10 promoter of the *icm* genes, were found upstream of eight *icm* genes [217]. Unlike *L. pneumophila*, which has between 91 and 400 bp of non-coding sequence upstream of genes that start transcriptional units within the T4BSS [217], the *C. burnetii* T4BSS has a more compact gene arrangement. Only *icmW*, *icmV*, *icmT*, *dotD*, *icmQ*, and *dotP* have more than 90 bp of non-coding sequence upstream, and none had more than 262 bp. Many of the linked genes have very short intervening sequences (single and low double digit). In

addition, the *C. burnetii* sequences upstream of *icmW*, *icmV*, *icmT*, *dotD*, *icmQ*, and *dotP* has no apparent bacterial (*E. coli*) consensus -10 sequences (TATAAT) or other readily recognizable regulatory elements (E. I. Shaw, unpublished observations).

Analysis of the *C. burnetii* RI locus shows the divergence of three gene groupings (Figure 1.1): *icmX*←CBU1651←*icmW*, *icmV*→*dotA*, and CBU1646←*dotB*←*dotC*←*dotD* ←*icmS*←*icmT*. As a result of the genetic orientation of these loci, the transcriptional regulatory mechanisms and products of these gene linkage groups (not precluding each individual ORF's regulation) are likely to be coordinately regulated. Characterization of the regulation of *C. burnetii*'s T4BSS RI will provide significant insight into the expression of this virulence mechanism during infection.

In *L. pneumophila*, the T4BSS is essential for secreting proteins involved in bacterial intracellular trafficking and replication within macrophages. As discussed, the *C. burnetii* NMI genome sequence revealed ORFs with significant homology and organized into two regions similar to those found in the *L. pneumophila* T4BSS. Currently, little else is known about the *C. burnetii* T4BSS regions and the role they play in establishing and/or maintaining infection.

CHAPTER II

SUMMARY SCOPE OF STUDY

AND ABSTRACT

Since its discovery in 1935 in both the United States and Australia, *C. burnetii* has come to be recognized as a naturally obligate intracellular pathogen that causes acute (Q fever) and chronic infections in humans [12]. Animal reservoirs of *C. burnetii* (particularly ruminants) can develop chronic infections and shed environmentally stable infectious particles that can be acquired by humans and other animals via inhalation [218, 219]. After inhalation, *C. burnetii* infects alveolar macrophages, where it replicates within a PV [122]. Once inside a host cell, *C. burnetii* exhibits a bi-phasic life cycle that starts with the environmentally stable SCV form and then changes into the metabolically active and replicative LCV form [120, 121]. While both forms are infectious in tissue culture models [128], SCVs are thought to be the primary form associated with naturally acquired infections [122].

Like many pathogenic intracellular bacteria, *C. burnetii* induces phagocytosis [168] and uses host cell materials (*e.g.*,, amino acids, nucleotides, etc.) to its advantage [167, 168, 220-222]. Upon infection, *C. burnetii* is trafficked along the endocytic pathway and eventually resides within vacuoles that retain many features of mature phagolysosomes [169, 179, 181, 223-226]. However, it has been shown that *C. burnetii* trafficking along the endocytic pathway is delayed for approximately two hours post infection (hpi) [177] and that as early as five minutes PI the *C. burnetii* PV appears to associate with the LC3 marker of the autophagy pathway [179, 180, 225]. After an initial 24 to 48 hour lag in replication, the organism replicates every 10 to 12 hours until approximately six days PI when asynchronous conversion back to SCVs begins within the PV [129] and continues until host cell lysis.

The enlargement and maintenance of the *C. burnetii* containing PV is dependent on *C. burnetii* proteins [187, 188]. Studies employing bacteriostatic concentrations of chloramphenicol, carbenicillin, and nalidixic acid to inhibit bacterial protein synthesis, cell wall synthesis, and DNA replication, respectively, demonstrated that *C. burnetii* specific effectors are involved in host-pathogen interactions [187, 188]. Infected cells treated with chloramphenicol early during infection contain small, tightly bound LAMP-1 positive PVs containing a single *C. burnetii* organism that dispersed throughout the host cell [187]. However, with removal of the chloramphenicol, vacuolar fusion reoccurred and resulted in spacious PVs containing multiple *C. burnetii* [188]. *C. burnetii* infected cells treated with carbenicillin or nalidixic acid were found to have mature spacious PVs containing multiple non-replicating *C. burnetii*, suggesting that vacuolar development requires metabolically active *C. burnetii* and is not dependant on bacterial density for complete PV maturation [187, 188]. Recent reports show a series of *C. burnetii* encoded Ankyrin repeat domain containing proteins that are secreted into the host cell cytoplasm [227, 228]. These proteins have been shown to associate with the PV membrane, microtubules, and mitochondria when expressed ectopically in eukaryotic cells [227]. In addition, *C. burnetii* actively mediates inhibition of host cell apoptosis by activating Akt and Erk1/2 [229], which presumably prolongs its opportunity for intracellular replication [229, 230].

Secretion systems specifically involved in virulence include the type III and type IV secretion systems (T4SS). Secretion systems have been found in a wide array of extracellular, facultatively intracellular, and obligate intracellular pathogens [191, 192, 196-198]. In particular, the T4SS category encompasses various adaptations of archaic

bacterial conjugation systems modified to export virulence factors. These factors include nucleoprotein complexes and effector proteins that secrete directly into a host or into the extracellular milieu [194, 196, 199]. At present, the type IV category has been subdivided into two families, (*i*) the VirB/D4 T4ASS and (*ii*) the Dot/Icm T4BSS [194].

The categorical T4ASS is found in *Agrobacterium tumefaciens* [194, 231, 232]. It has been shown to be used for direct injection of effector molecules into adjacent cells [194] or into the extracellular environment [233, 234]. These attributes of the T4ASS are a major virulence mechanism and a key factor in the molecular pathogenesis of bacteria possessing these systems [199, 232, 235]. T4ASS are composed of up to twelve genes encoding a pilus-like structure that transverses the inner membrane, periplasmic space, and outer membrane of Gram-negative bacteria [231]. Localization analyses of the *A. tumefaciens* T4ASS indicated that it is expressed polarly on the cell surface [236-239].

The *L. pneumophila* genome encodes an Lvh system, which has significant homology to the T4ASS system of *A. tumefaciens* [199]. Although shown to be present and expressed, the T4ASS of *L. pneumophila* has little affect on its virulence [240]. Alternately, *L. pneumophila* encodes a T4BSS that has been shown to be essential for its survival and pathogenesis within the host cell. The Dot/Icm system of *L. pneumophila* best characterizes the second subdivision of the type IV secretion system family, T4BSS [194]. This system is composed of 26 *dot/icm* genes that form the T4BSS complex encoded in two distinct pathogenicity islands designated RI and RII. As with the *A. tumefaciens* T4ASS, localization studies of the *L. pneumophila* T4BSS indicate polar expression of specific secreted substrates [210, 241, 242] and components [243] of the T4BSS.

Analysis of the *C. burnetii* RSA 493 (Nine Mile phase I strain) sequence revealed loci with significant homology and gene organization to both RI and RII of the *L. pneumophila* T4BSS [137]. This information extended previous work that had identified randomly sequenced *C. burnetii* fragments with similarity to *L. pneumophila* T4BSS genes [216]. The genome sequence, combined with studies using *C. burnetii* T4BSS analogs (IcmW, DotB, IcmS, and IcmT) to complement the respective *L. pneumophila* mutants [214, 215], led to the assumption that *C. burnetii* utilizes a T4BSS that is functionally akin to the *L. pneumophila* system. Studies have shown the *L. pneumophila* T4BSS is required for intracellular survival, effector protein secretion, and replication within host cells [202, 203, 244-248], indicating its vital role for *L. pneumophila* pathogenesis. The descriptive functionality of the T4BSS system in *C. burnetii* is lacking.

Expression studies of the *C. burnetii* T4BSS gene homologs have been limited both in the number of gene homologs analyzed as well as the breadth of the temporal analysis. One study analyzed total RNA collected 24, 48, and 96 hpi from cultured cells infected with *C. burnetii* Nine Mile phase II using reverse transcriptase PCR (RT-PCR) and found that gene specific transcripts for *icmS*, *icmQ*, and *dotB* were detectable by 48 hpi while *icmW* transcript was detectable as early as 24 hpi [214]. Other data showed that *icmS* and *dotC* transcripts were present by 8 hpi, with *dotB* transcripts present by 24 hpi, and *icmW* transcript present as early as 6 hpi [249, 250]. An additional study using quantitative reverse transcriptase PCR (RT-qPCR) demonstrated that a *dotA* transcript is detectable by 8 hpi with a peak in expression per genome equivalent at 72 hpi [129].

The *C. burnetii* T4BSS RI loci contains twelve genes (CBU1652 – CBU1641), nine of which are obvious homologs of *L. pneumophila* T4BSS genes [137]. Figure 1.1 shows a physical map of the twelve *C. burnetii* genes: (*i*) *icmX*←CBU1651←*icmW*, (*ii*) *icmV*→*dotA*→CBU1647, and (*iii*) CBU1646←*dotB*←*dotC*←*dotD*←*icmS*←*icmT*.

Considering the infectious life cycle of *C. burneti*, the genetic arrangement of the T4BSS loci in *C. burnetii*, and the importance of the T4BSS as a virulence factor in other bacterial pathogens, I sought to address the following questions:

1. Is the transcription and translation of the *C. burnetii* T4BSS RI genes temporally regulated throughout the course of the infectious cycle?
2. Is the *C. burnetii* T4BSS localized during infection of a host cell?

ABSTRACT

In nature, *Coxiella burnetii* is an obligate intracellular bacterium and the causative agent of acute Q fever. This pathogen invades eukaryotic host cells where it is able to reside and replicate within a PV that has attributes of a phagolysosome. Analysis of the *C. burnetii* NMI genome revealed ORF's with significant homology to the *L. pneumophila* T4BSS, a known virulence mechanism. In *C. burnetii*, little is known about the expression and sub-cellular localization of T4BSS proteins and the role they play in establishing and/or maintaining an infection. Here, temporal expression analysis of both mRNA and protein expression/ultra-structure for the *C. burnetii* T4BSS Region I gene homologs was analyzed during infection of host cells. First, I demonstrated the efficacy of a novel technique to isolate enriched bacterial specific RNA from infected cells. This method resulted in up to 1,300 fold increase in *C. burnetii* specific RNA compared to traditional TRI Reagent lysis methods. Subsequently, the *C. burnetii* RI T4BSS genes were shown to be expressed in three transcriptionally linked groups which quantitative PCR then demonstrated to be expressed at the highest levels early during infection. This correlates to temporally expression changes in these genes as *C. burnetii* is trafficked early during infection and shifts from SCV→LCV forms of the bacteria. Next, I found that *C. burnetii* IcmT increased significantly ($p < 0.05$) from 0 to 24 hours post infection (hpi), and was then maintained from 24 to 168 hpi. Additionally, I demonstrate that the *C. burnetii* T4BSS is expressed polarly on the *C. burnetii* LCV form by 8 hpi and remains polar through 168 hpi. These data led me to conclude that the *C. burnetii* T4BSS RI is temporally regulated at both the RNA and protein level and that the T4BSS is polarly expressed on the LCV form of the bacteria.

CHAPTER III

A METHOD OF ISOLATING ENRICHED BACTERIAL RNA FROM CELLS INFECTED WITH THE OBLIGATE INTRACELLULAR BACTERIUM *COXIELLA BURNETII*

The obligate intracellular bacterium *Coxiella burnetii* is the causative agent of Q fever, a human illness that is normally acquired through an aerosol route and manifests with flu-like symptoms [12]. *C. burnetii* invades a host cell, is trafficked along the endocytic pathway, establishes residence, and replicates intracellularly within a membranous bound parasitophorous vacuoles (PV) with characteristics of a phagolysosome [178]. The obligate nature of *C. burnetii* during infection makes acquiring enriched or purified *C. burnetii* RNA which is representative of *in vivo* RNA expression a challenge due to (*i*) the time required to isolate intracellular bacteria from host cells prior to RNA isolation, and (*ii*) the short half-life of bacterial RNA. For these reasons *C. burnetii* RNA expression studies have primarily been performed using total RNA from *C. burnetii* infected host cells where the vast majority of the harvested RNA is eukaryotic [214]. This limits the downstream techniques which can be employed for *C. burnetii* specific gene expression studies, most notably Northern blot and RNA microarray analysis. While the growth of *C. burnetii* in a cell-free environment was recently demonstrated [251], this significant achievement does not eliminate the need to develop means of analyzing the gene expression of these bacteria in their intracellular environment.

Commonly used *C. burnetii* purification methods have included osmotic lysis [252], cell homogenizing/disruption [253, 254], and sonic disruption of cells [173], which can result in the generation of bacterial aerosols. Considering the risks involved with *C. burnetii*'s manipulation and the difficulty purifying *C. burnetii* away from host cell material, new methods to safely and efficiently purify this pathogen are significant. The observation that cholesterol is integral to the *C. burnetii* containing PV membrane [223],

while absent from the *C. burnetii* membrane, led to the recent development of a digitonin based *C. burnetii* purification method [255]. This method is advantageous as it occurs in a closed system, providing a safe alternative to traditional *C. burnetii* harvesting techniques [255]. This method has proven successful for the isolation of infectious *C. burnetii* [255] and recently has been employed in our laboratory to isolate infectious *Rickettsiae* (unpublished data). However, the possibility of modifying this method to produce stable RNA samples which are enriched for *C. burnetii* RNA has not been explored.

I co-developed a new protocol for isolation of RNA from intracellular *C. burnetti* cells in human cell lines. The method involves key modifications of the sucrose phosphate digitonin (SPD) lysis method [255], specifically, the addition of GeneLock™ (Sierra Molecular Inc., Sonora, CA), a DNA/RNA stabilizing solution [256], to protect the *C. burnetii* RNA during the bacterial enrichment process and performing the entire protocol on ice. I compare the level of bacterial RNA enrichment in parallel RNA samples that were isolated from infected cells using this approach (which I describe as SPD-GL/ice) to: 1) bacteria enriched by SPD lysis at room temperature (SPD/rt) as previously isolated [255], 2) bacteria enriched by SPD lysis on ice (SPD/ice), 3) SPD lysis with 20% GeneLock™ in the lysis solution at room temperature (SPD-GL/rt), to demonstrate the level of enrichment of the bacterial fraction as well as the stability of the RNA obtained using this SPD-GL/ice approach.

Confluent Vero cells (CCL-81, American Type Culture Collection) were infected with *C. burnetii* Nine Mile, Phase II, clone 4 RSA439 (CBII), at a genome equivalent MOI of 100 and allowed to propagate in RPMI – 1640 medium (HyClone®, Logan, UT)

supplemented with 5% fetal bovine serum (Atlanta Biologicals, Lawrenceville, GA) in an atmosphere of 5% CO_2 at 37°C with regular media changes. Infected cells were maintained until an infection rate of ~70% was observed by phase contrast light microscopy at ~3 weeks post infection. The *C. burnetii*-infected Vero (CBII/Vero) cells were then trypsinized (Mediatech, Inc., Herndon, VA) and split into T-75 cm^2 cell culture flasks for all downstream experiments described below. The CBII/Vero cells were then allowed to propagate to confluency (approximately 2 days) reaching an infection rate of >90% as observed by indirect immunofluorescence antibody (IFA) assay. A representative IFA is shown in Figure 3.1.

Enrichments were performed by removing the culture medium from infected monolayers, followed by washing cells once with SP buffer (250 mM sucrose, 12.8 mM KH_2PO_4, 72.6 mM NaCl, 53.9 mM Na_2HPO_4 at pH 7.4) to remove residual medium. SPD-GL buffer (SP buffer containing digitonin at 0.2 mg ml^{-1} and GeneLock™ solution added to 20% of the final volume) was then added (10ml per T-75 cm^2 culture flask). Flasks were incubated on ice for 30 min. with moderate rocking during which time cell lysis occurs [255]. Cell lysates were then collected into 15 ml conical tubes and centrifuged at 1,200 × g for 15 min at 4 °C to pellet host cellular debris. Supernatants were then transferred to new 15ml tubes and centrifuged for 10min. at 13,000 × g at 4°C to pellet the released *C. burnetii*. The supernatant fluid was discarded and the *C. burnetii* pellets were solubilized in 1ml of TRI Reagent® Solution (Ambion, Austin, TX), transferred to RNase free screw cap microcentrifuge tubes and stored at -80°C until RNA extraction.

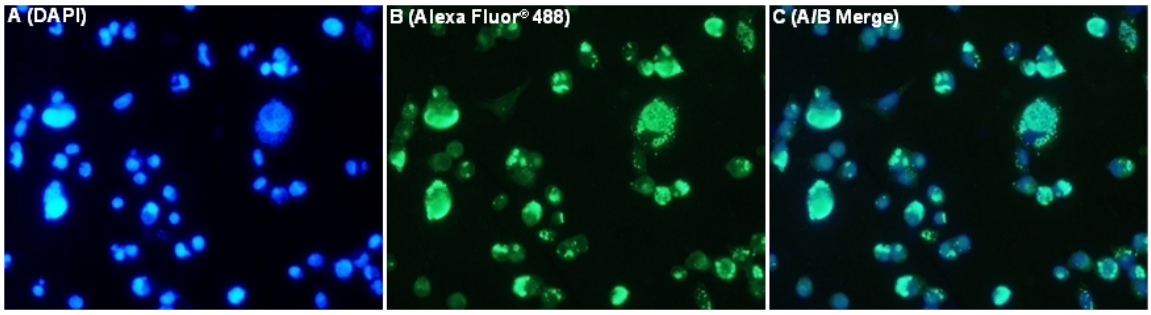

Figure 3.1: IFA assay of *Coxiella burnetii* NMII infected Vero cells. Panel series A-C are 3-week post infection single field IFA micrograph images. Panel A, DAPI (Sigma, St. Louis, MO) stained cell nuclei (blue). Panel B, *C. burnetii* cells (green), primary rabbit antibody against whole-killed *C. burnetii* NMII with secondary goat anti-rabbit IgG Alexa Fluor® 488 conjugated antibody (Molecular Probes, Eugene, OR). Panel C, merged A/B image (Paint.NET v3.35), showing >90% *C. burnetii* NMII infection of Vero cells. Micrograph images were captured via a Nikon DS FI1 camera on a Nikon Eclipse TE 2000-S microscope at 40X UV DIC.

For comparative purposes, this procedure was repeated using parallel CBII/Vero cells along with SPD lysis buffer with GeneLock™ at room temperature (SPD-GL/rt), with SPD lysis buffer with no GeneLock™ (SPD/rt), and with SPD lysis buffer with no GeneLock™ on ice (SPD/ice).

Total RNA from each of the crude *C. burnetii* pellets produced using the SPD and SPD-GL enrichment methods was isolated following standard TRI Reagent® Solution protocols (Ambion). All total RNA preparations were then DNased for one hour with RQ1 DNase (Promega, Madison, WI), followed by phenol extraction and nucleic acid precipitation. The resulting precipitates were resuspended in nuclease free H_2O. To further remove small DNA and RNA species, single nucleotides, and potential protein residue, each sample was lithium chloride precipitated following the Ambion Lithium Chloride Precipitation solution protocol (Ambion Technical sheet 9480). Each RNA sample was then confirmed as DNA free via PCR analysis using GoTaq PCR Master Mix (Promega, Madison, WI) and primers CB652 (forward) 5'–ACGGGTGAGTAATGCGTAGG and CB653 (reverse) 5'–GGGCTTTCACATCCGACTTA [257] designed against the *C. burnetii* 16S *rrs* ribosomal gene. To demonstrate integrity of the RNA, RNA samples (300ng/sample) from each isolation method was analyzed using an Agilent 2100 Bioanalyzer On-chip gel electrophoresis analysis system (Agilent Technologies, Inc., Waldbronn, Germany) and the microfluidic RNA 6000 Nano Labchip® kit (Agilent Technologies, Inc.).

The level of enrichment of bacterial RNA in all above procedures was compared to total, un-enriched RNA harvested directly from CBII/Vero monolayers using TRI Reagent® (Ambion) according to the manufacturer's instructions (referred to here as TR

samples). In order to quantify the relative enrichment of *C. burnetii* vs. Vero cell RNA from each isolation method, I used reverse transcriptase quantitative PCR (RT-qPCR) analysis and the $2^{-\Delta\Delta CT}$ relative fold comparison method to compare Vero 18S and *C. burnetii rrs* (16S) rRNA cycle threshold (C_T) values. qPCR primer sets for (*a*) eukaryotic 18S and (*b*) *rrs* were: (*a*) Hs18S-F (forward) 5' – CATTCGAACGTCTGCCCTAT, Hs18S-R (reverse) 5' – CAATTACAGGGCCTCGAAAG [257], (*b*) Q16s-F (forward) 5' – CCATGAAGTTGGAATCGCTAG (forward), Q16s-R (reverse) 5' – ACTCCCATGGTGTGACGG [129]. Both primer sets were tested to determine primer efficiency and found to be within the efficiency window for the $2^{-\Delta\Delta CT}$ relative fold calculation method [258]. Single step RT-qPCR analysis utilizing SuperScript™ III (Invitrogen, Carlsbad, CA) reverse transcriptase and the SYBR Green Master Mix Kit (Applied Biosystems, Foster City, CA) was performed on an ABI 7500 cycler using 10ng of total RNA per reaction. To calculate the fold enrichment values for sample sets the 18S gene C_T values were used as the primary reference standard (1° ΔC_T), with the *C. burnetii rrs* gene C_T values functioning as the internal secondary reference (2° ΔC_T) for each sample set respectively. Fold enrichment values were then compared to each other to determine the relative ratio of fold differences between each RNA isolation method. Statistical significance between the methods was evaluated using the single factor ANOVA method with a 95% confidence interval by MS Excel 2007 (Microsoft, Redmond, WA). To determine statistical significance, a minimum of two biological replicates having no less than four technical replicates each were used.

Figure 3.2 shows a representative densitometry plot image of the corresponding chromatographic data for each sample. Bands representative of eukaryotic 18S and 28S

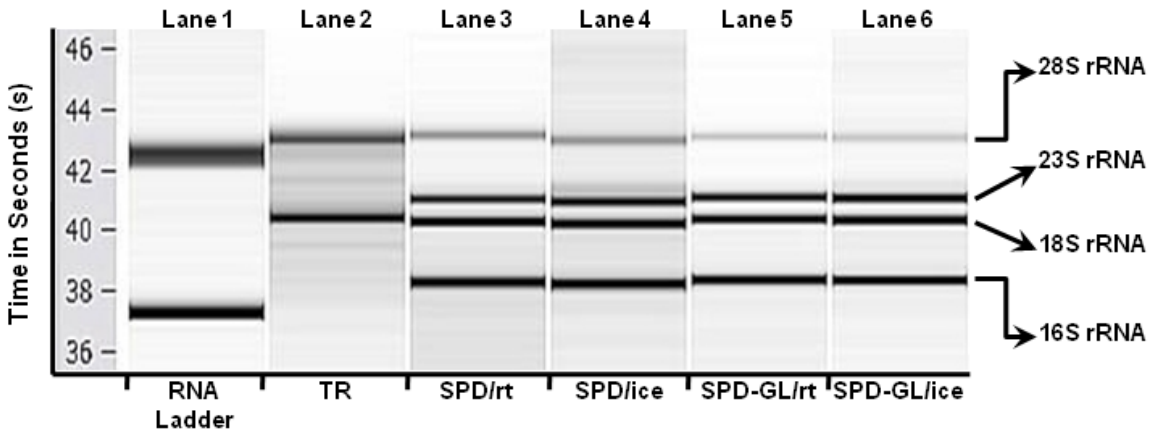

Figure 3.2: Agilent 2100 Bioanalyzer On-chip gel electrophoresis densitometry plot of CBII/Vero RNA. Lane 1- Agilent RNA Ladder, Lane 2- TR isolated total RNA, Lane 3- SPD/rt enriched RNA, Lane 4- SPD/ice enriched RNA, Lane 5- SPDGL/rt enriched RNA, Lane 6- SPD-GL/ice enriched RNA. Lanes 2 – 6 show RNA integrity, while lanes 3 – 6 indicate a significant *C. burnetii* RNA enrichment as the prokaryotic 16S and 23S rRNA bands become apparent relative to the eukaryotic 18S and 28S rRNA bands.

rRNA can be seen in Figure 3.2, lanes 2-6, with little to no non-specific/degraded RNA, demonstrating the integrity of the RNA samples produced by all methods. The appearance of prokaryotic 16S and 23S rRNA bands in Figure 3.2, lanes 3 – 6, suggests that *C. burnetii* RNA is substantially enriched by the SPD and SPD-GL methods relative to the TR sample (lane 2). This qualitative evidence indicates an enrichment of *C. burnetii* RNA species occurs within a given total RNA sample following the crude enrichment of *C. burnetii* by the SPD and SPD-GL methods when compared to a standard RNA isolation technique.

A quantitative measure of the relative enrichment of *C. burnetii* RNA following bacterial enrichment compared to total RNA harvested directly from infected cells is depicted in Figure 3.3. When comparing equal amounts of total RNA, the quantitative data indicate that the SPD-GL/ice method enriches *C. burnetii rrs* RNA to a 1,377:1 ratio relative to TR isolation, while the SPD-GL/rt to TR ratio is 366:1. In analyzing the enrichment without GeneLock™, it was found that the SPD/ice to TR *C. burnetii* RNA ratio was 350:1, while the SPD/rt to TR ratio was 26:1. These data indicate that significant increases exist in the relative amount of *C. burnetii* RNA to Vero cell RNA after digitonin lysis and differential centrifugation. Comparing the enrichment method with or without GeneLock™ in the solution indicate its usefulness in stabilizing RNA. Figure 3.3 shows that the SPD-GL/ice to SPD/ice ratio is ~4:1, while the SPD-GL/rt to SPD/rt ratio is 14:1. Comparing the effects of performing the enrichments on ice vs. rt, indicates that there is a 3.7:1 ratio when comparing SPD-GL/ice relative to SPD-GL/rt, and a 13.5:1 ratio when comparing SPD/ice to SPD/rt. These calculations indicate that (*i*) performing the enrichment steps on ice increases RNA stability and yield, (*ii*) that using

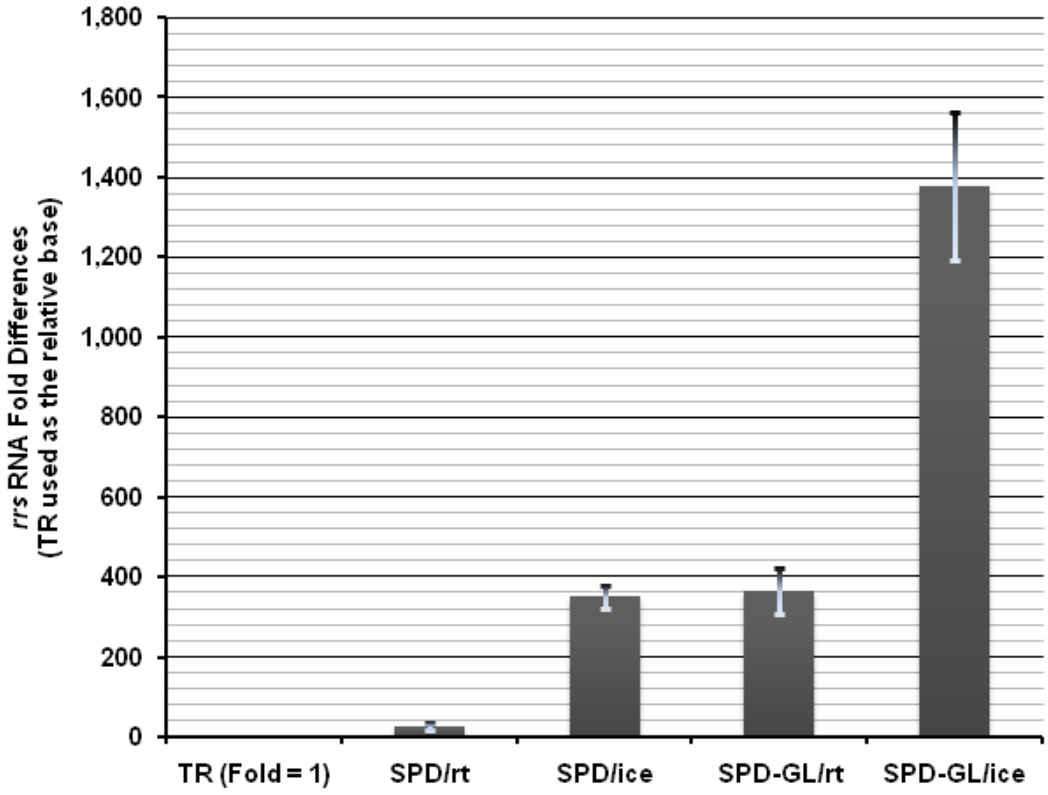

Figure 3.3: Fold enrichment of *C. burnetii rrs* RNA harvested by various methods. RT-qPCR using 10ng of RNA samples isolated by TR, SPD, or SPD-GL methods at room temperature (rt) or on ice. Relative *C. burnetii rrs* fold values are as follows: TR acts as the relative standard and yields a fold value of 1, SPD/rt yields a 26 (error +9), SPD/ice a 350 (error + 28), SPD-GL/rt a 366 (error + 56) and SPD-GL/ice a 1,377 (error + 186) relative fold increase(s) to the TR standard.

GeneLock™ during the enrichment is roughly equivalent to performing it on ice alone, and (*iii*) performing the enrichment on ice using GeneLock™ provided the greatest relative *C. burnetii* RNA enrichment. Using single factor ANOVA at 95% confidence interval, significant differences were confirmed between all comparative methods analyzed except when comparing the SPD-GL/rt relative to the SPD/ice methods (approximate 1:1 comparative fold value ratio). Overall, these data indicate that the utilization of SPD-GL/ice yields an increase of *C. burnetii rrs* RNA relative to either SPD-GL/rt or SPD/ice at an approximate 4:1:1 ratio.

The ability to isolate total RNA from infected cell culture that is enriched for the bacterial fraction may expand the downstream techniques available for expression analysis. Commercial bacterial RNA enriching products such as Microbe Enrich (Ambion) has been reported for isolating the RNA of obligate intra-cytoplasmic *Rickettsiae* spp. [256, 259]. However, these products may be cost prohibitive, particularly when analyzing multiple growth conditions and time points or when obtaining large scale amounts of RNA. Using SPD-GL/ice to enrich for obligate intracellular bacteria prior to RNA isolation allows experiments to be scaled-up for the acquisition of enriched RNA from very large numbers of infected cells. In addition, other than the digitonin and GeneLock™, the buffers are composed of chemicals common to molecular biology laboratories.

In summary, I have demonstrated the development of a simple, safe, inexpensive method to significantly enrich the RNA of *C. burnetii* harvested from infected host cells by modifying a recently described digitonin lysis method [255] with the addition of the nucleic acid stabilizing solution GeneLock™ to the process. In addition, I found that

performing the entire process on ice further enhances the enrichment. While there is undoubtedly some *C. burnetii* RNA lost during the procedure, the quality of the SPD-GL/ice enriched RNA may prove useful for *in vivo* RNA expression analysis techniques where total RNA with relatively lower amounts of host cell RNA would be advantageous. The method described here may well be applicable to the isolation of enriched bacterial RNA from a broad range of obligate intracellular bacteria.

CHAPTER IV

EXPRESSION ANALYSIS OF *COXIELLA BURNETII* (NMII) TYPE IVB SECRETION SYSTEM REGION I GENES DURING INFECTION OF HOST CELLS

Coxiella burnetii is an obligate intracellular pathogen that causes acute (Q fever) and chronic infections in humans [12]. Animal reservoirs of *C. burnetii* (particularly ruminants) can develop chronic infections and shed environmentally stable infectious particles that can be acquired by humans and other animals via inhalation [218, 219]. After inhalation, *C. burnetii* infects alveolar macrophages, where it replicates within a parasitophorous vacuole (PV) [122]. Once inside a host cell, *C. burnetii* exhibits a bi-phasic life cycle which starts with the environmentally stable small cell variant (SCV) form, then changes into the metabolically active and replicative large cell variant (LCV) form [120, 121]. While both forms are infectious in tissue culture models [128], SCVs are thought to be the primary form associated with naturally acquired infections [122].

Like many pathogenic intracellular bacteria, *C. burnetii* induces phagocytosis [168] and uses host cell materials (*e.g.*, amino acids, nucleotides, etc.) to its advantage [167, 168, 220-222]. Upon infection, *C. burnetii* is trafficked along the endocytic pathway and eventually resides within vacuoles that retain many features of mature phagolysosomes [169, 179, 181, 223-226]. However, it has been shown that *C. burnetii* trafficking along the endocytic pathway is delayed for approximately two hours post infection (hpi) [177] and that as early as five minutes post infection the *C. burnetii* PV appears to associate with the LC3 marker of the autophagy pathway [179, 180, 225]. After an initial 24-48 hour lag in replication, the organism replicates every ten to twelve hours until approximately six days post infection when asynchronous conversion back to SCVs begins within the PV [129], and continues until host cell lysis.

The enlargement and maintenance of the *C. burnetii* containing PV is dependent on *C. burnetii* proteins [187, 188]. Studies employing bacteriostatic concentrations of

chloramphenicol, carbenicillin, and nalidixic acid to inhibit bacterial protein synthesis, cell wall synthesis, and DNA replication, respectively, demonstrated that *C. burnetii* specific effectors are involved with host-pathogen interactions [187, 188]. Infected cells treated with chloramphenicol early during infection contained small tightly bound LAMP-1 positive PVs containing single *C. burnetii* that were dispersed throughout the host cell [187]. However, with the removal of the chloramphenicol, vacuolar fusion reoccurred and resulted in spacious PVs containing multiple *C. burnetii* [188]. *C. burnetii* infected cells treated with carbenicillin or nalidixic acid were found to have mature spacious PVs containing multiple non-replicating *C. burnetii*, suggesting that vacuolar development requires metabolically active *C. burnetii* and is not dependant on bacterial density for complete PV maturation [187, 188]. Recent reports show a series of *C. burnetii* encoded Ankyrin repeat domain containing proteins are secreted into the host cell cytoplasm [227, 228]. These proteins have been shown to associate with the PV membrane, microtubules, and mitochondria when expressed ectopically within eukaryotic cells [227]. In addition, *C. burnetii* actively mediates inhibition of host cell apoptosis by activating Akt and Erk1/2 [229], which presumably prolongs its opportunity for intracellular replication [229, 230].

Secretion systems specifically involved in virulence include the type III and type IV secretion systems (T4SS). These systems have been found in a wide array of extracellular, facultatively intracellular, and obligate intracellular pathogens [191, 192, 196-198]. The T4SSs has been subdivided into two groups, the type IVA secretion system (T4ASS), encoded by the *virB* operon [199], and the type IVB secretion systems (T4BSS) [248, 260, 261]. The best-characterized T4BSS exists in *Legionella*

pneumophila, where it is also known as the *dot/icm* system [202, 216, 248, 260, 261]. While the T4ASS and the T4BSS share some homologies, T4ASS have 12 or fewer ORFs while the T4BSS have 23 – 26 ORFs [137, 199]. *L. pneumophila's* T4BSS has been shown to be essential for effector protein secretion, bacterial intracellular trafficking, and replication within macrophages as well as amoeba [202, 203, 244-247]. Analysis of the *C. burnetii* RSA 493 (Nine Mile phase I strain) genome sequence revealed loci with significant homology and gene organization to both Region I and Region II of the *L. pneumophila* T4BSS [137]. This information extended previous work which had identified randomly sequenced *C. burnetii* fragments with similarity to *L. pneumophila* T4BSS genes [216]. The genome sequence, combined with studies using *C. burnetii* T4BSS analogs (IcmW, DotB, IcmS, and IcmT) to complement the respective *L. pneumophila* mutants [214, 215], led to the assumption that *C. burnetii* utilizes a T4BSS that is functionally akin to the *L. pneumophila* system. Gene expression studies of the *C. burnetii* T4BSS homologs have been limited both in the number of homologs analyzed as well as the breadth of the temporal analysis. One study analyzed total RNA collected 24, 48, and 96 hpi from cultured cells infected with *C. burnetii* Nine Mile phase II (NMII) using reverse transcriptase PCR (RT-PCR) and found that gene specific transcripts for *icmS*, *icmQ*, and *dotB* were detectable by 48 hpi while *icmW* transcript was detectable as early as 24 hpi [214]. Other data showed that *icmS* and *dotC* transcripts were present by 8 hpi, with *dotB* transcripts present by 24 hpi, and *icmW* transcript present as early as 6 hpi [249, 250]. An additional study using quantitative reverse transcriptase PCR (RT-qPCR) demonstrated that *dotA* transcript is detectable by 8 hpi with a peak in expression per genome equivalent at 72 hpi [129].

To more fully characterize the transcriptional and translational regulation of the *C. burnetii* T4BSS, I analyzed the RNA expression profile of the Region I (RI) genes at numerous points over a time course of infection. The *C. burnetii* T4BSS RI loci contain twelve genes (CBU1652 – CBU1641), nine of which are *L. pneumophila* T4BSS homologs [137]. As shown in Figure 4.1 (a), the orientations of the twelve *C. burnetii* genes are suggestive of three operons: (*i*) *icmX*←CBU1651←*icmW*, (*ii*) *icmV*→*dotA*→CBU1647, and (*iii*) CBU1646←*dotB*←*dotC*←*dotD*←*icmS*←*icmT*. To assess the temporal regulation of *C. burnetii* T4BSS RI homologs during the course of the infectious cycle, synchronous *C. burnetii* infection of host cells using purified SCVs [129] was employed, followed by RT-PCR and RT-qPCR using RNA samples purified from infected host cells at early, mid, and late stages of infection. I identified transcriptional linkages between the *C. burnetii* T4BSS RI homologs and defined their relative expression levels during an infectious cycle. Additionally, the correlation between protein and RNA expression of IcmT was assessed to determine relative protein expression during the infectious cycle.

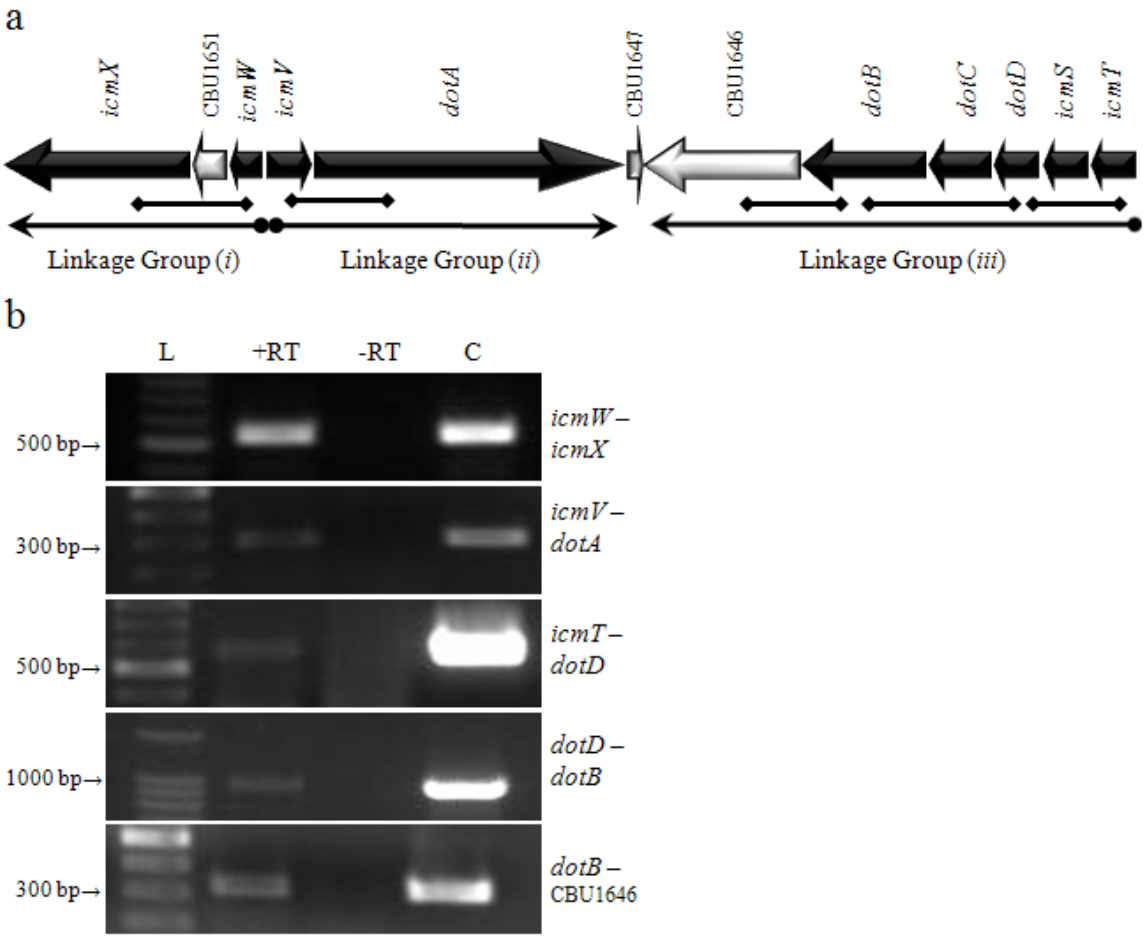

Figure 4.1: *Coxiella burnetii* Type IVB Secretion System Region I gene map. Panel a. Physical map of *C. burnetii* T4BSS RI. Solid arrows represent T4BSS homologs. Open arrows represent non-T4BSS ORFs. Gene designations are indicated above each ORF. Panel b. Agarose gel image(s) showing RT-PCR products corresponding to co-transcribed genes from Linkage Group *i*, *ii*, and *iii* (correlating to the diamond tipped lines in Panel a). L, 100 bp DNA ladder (size designated on left). +RT, with reverse transcriptase. –RT, without reverse transcriptase. C, DNA control.

MATERIALS AND METHODS

Bacterial Cultivation and Purification. *C. burnetii* Nine Mile Phase II Clone 4 (NMII) was propagated in African green monkey kidney (Vero) cells in RPMI medium with 5% fetal bovine serum (FBS) and the SCV form of the organism was isolated as previously described [129]. The SCV isolation was performed using digitonin lysis of host cells followed by low speed and high speed differential centrifugation to isolate *C. burnetii* cells similar to published methods [255]. Briefly, *C. burnetii* infected Vero cells were rinsed once with sucrose phosphate (SP) buffer, followed by lysis of the eukaryotic cells in SP buffer (pH 7.4), 0.2mg/ml digitonin. The lysate was then removed and subjected to a 4,000 × g centrifugation to pellet host cell debris. The supernatant containing *C. burnetii* SCVs was then subjected to 12,000 × g centrifugation to pellet the SCVs. The crude SCV pellet was then resuspended in SPG buffer (0.7M sucrose, 3.7mM KH_2PO_4, 6.0mM K_2HPO_4, 0.15M KCl, 5.0mM glutamic acid, pH 7.4) and stored at -80°C. Organisms were enumerated by genome equivalents using qPCR [138].

Cell culture and infection. Vero cells were propagated in RPMI 1640 media containing 5% FBS with gentamicin (20 µg/ml) at 37°C and 5% CO_2. Culture media without antibiotics was added two hours prior to bacterial infection that was carried out in the same media. Vero cells were infected with *C. burnetii* NMII at an MOI of 0.5, resulting in 40% infection. After two hours (designated as time 0), the inoculum was removed, the cells were washed once with PBS and infected cultures incubated in RPMI, 5% FBS at 37°C, and 5% CO_2 for periods of 0, 8, 16, 24, 36, 48, 96, and 168 hpi. To determine *de novo* synthesis of *C. burnetii* RNA upon infection of Vero cells, cultures

parallel infections were either treated with the RNA synthesis inhibitor rifampin (+Rif) at 20µg/ml in the culture media or mock treated (-Rif) and harvested for total RNA.

RNA isolation and quality control. Total RNA was harvested from infected tissue cultures at various times post-infection using TRI Reagent® (Ambion, San Antonio, TX). In some cases, enriched *C. burnetii* RNA was isolated using a modification of the digitonin based bacterial isolation method [255]. The addition of GeneLock™ (Sierra Molecular, Sonora, CA) to 20% in the lysis solution was found to protect the integrity of the RNA during bacterial enrichment while substantially enriching the relative amount of *C. burnetii* specific RNA in a given sample (J. K. Morgan and E. I. Shaw, submitted). All RNA samples were DNase treated to remove contaminating DNA with RQ1 DNase (Promega, Madison, WI).

RNA analysis. RT-PCR analysis was carried out using the Access Quick RT-PCR Kit (Promega) following the manufacturer's instructions. All oligonucleotide primers used in this study (Integrated DNA Technologies, Coralville, IA) are shown in Table 4.1. Forward [f] and reverse [r] primer pairs: CB58 [f] and CB57 [r] (*icmW* – CBU1651 – *icmX*), CB59 [f] and CB60 [r] (*icmV* – *dotA*), CB603 [f] and CB602 [r] (*dotB* – CBU1646), CB63 [f] and CB64 [r] (*dotD* – *dotC* – *dotB*), and CB62 [f] and CB61 [r] (*icmT* – *icmS* – *dotD*) were used to demonstrate transcriptional linkage. Full-length RT-PCR analysis of *icmT*, *icmV*, and *icmW* was performed using CB78 [f] and CB79 [r], CB70 [f] and CB71 [r], and CB40 [f] and CB41 [r], respectively.

Oligonucleotide primers (Table 4.1) for RT-qPCR analysis of *icmX*, *icmW*, *icmV*, *dotA*, *dotB*, and *icmT* were designed using Primer3Plus [257]. The *C. burnetii rrs* (16S) specific primers used were the same as previously published [129]. All primer sets were

TABLE 4.1: Oligonucleotide primers used in this study.

Gene Target	*RefSeq ID*	*Designation*	*Gene Primer Sequence* (5'→3')	*Purpose*
rrs[1]	CBU 16S	Q16s-F[f]	CCATGAAGTTGGAATCGCTAG	RT-qPCR
		Q16s-R[r]	ACTCCCATGGTGTGACGG	RT-qPCR
icmT	CBU 1641	CB62[f]	GCAAAATCGCCATAGCATGGTG	RT-PCR
		CB578[f]	GGGATGGCAAAACAGCCTTT	RT-qPCR
		CB579[r]	CCGTCACCGCTACGATGAG	RT-qPCR
		CB78[f]	[2]*CACC*ATGAAATCTCTCGATGAGG	PCR, RT-PCR
		CB79[r]	[3]*TTA*GTTATCCCACCATGCTATGG	PCR, RT-PCR
dotD	CBU 1643	CB63[f]	TCCAGACGGATCATTGAGC	RT-PCR
		CB61[r]	CAACGCCAGAAAGAGGGGCAGC	RT-PCR
dotB	CBU 1645	CB64[r]	CAATGTGTTTGGGTTCGAAGCG	RT-PCR
		CB603[f]	ACTCGACAGTGATCCCGAAC	RT-PCR
		CB586[f]	CCACGGGTTCGGGTAAAAG	RT-qPCR
		CB587[r]	GCGCTTCGGCCAATTCT	RT-qPCR
glycine betaine transporter[4]	CBU 1646	CB602[r]	TTACCCAGCGGCGTTAATAC	RT-PCR
dotA	CBU 1648	CB60[r]	CGATAATGCCTTCATTGAGC	RT-PCR
		CB654[f]	ACAATCAATCCCCGTTGAAA	RT-qPCR
		CB655[r]	AGCTATCATCGCCTGGCTTA	RT-qPCR
icmV	CBU 1649	CB59[f]	TTCCAAATGAAGCAACGC	RT-PCR
		CB592[f]	TGGCGGGTTACACGGTTT	RT-qPCR
		CB593[r]	CGCAGACGAAAGCCGATAA	RT-qPCR
		CB70[f]	[2]*CACC*ATGATTCTTTTGGAGTCTTCC	RT-PCR
		CB71[r]	[3]*TTA*TTGTTTGGACCCCTTAAAGGTG	RT-PCR
icmW	CBU 1650	CB58[f]	CAAACTCTTGAGGAAGG	RT-PCR
		CB594[f]	CGCCGCTGCGAAAGTG	RT-qPCR
		CB595[r]	ACCGGCGGTGTCTATTTCC	RT-qPCR
		CB40[f]	[2]*CACC*ATGCCAGATCTGTCGC	RT-PCR
		CB41[r]	[3]*TTA*TAAACCACCTTCCTCAAGAG	RT-PCR
icmX	CBU 1652	CB57[r]	GAAGCAATACCAAGAACACG	PCR, RT-PCR
		CB658[f]	CCGCTTATAATTCGGACCAA	RT-qPCR
		CB659[r]	TTGATAAGCGGGATTGTTCA	RT-qPCR

[1] *rrs*, primers designed by Coleman, S.A., et al., 2004 [129].
[2] *CACC*, non-*C. burnetii* sequence, engineered directional cloning sequence for pET200/D-TOPO plasmid.
[3] *TTA*, non-*C. burnetii* sequence, engineered stop codon.
[4] glycine betaine transporter, Beare, P.A., et al., 2009 (supplemental data table) [262].
[f, r,] designate Forward and Reverse primers respectively.

empirically tested to determine primer efficiency and found to be within the efficiency window for the $2^{-\Delta\Delta CT}$ relative fold calculation method [258, 263]. Single step RT-qPCR analysis using SuperScript III (Invitrogen, Carlsbad, CA) reverse transcriptase and the SYBR Green Master Mix Kit (Applied Biosystems, Foster City, CA) was performed on an ABI 7500 cycler. Each reaction contained a total of 15µL and included 20ng of total RNA. To determine the calculated fold values for sample sets, the *C. burnetii rrs* C_T values were used as the primary reference standard (1° ΔC_T), with respect to each gene of interest as the internal secondary reference (2° ΔC_T) for each RNA sample set, respectively. Statistical significance between the time points was evaluated by single factor ANOVA with a 95% confidence interval using MS Excel 2007 (Microsoft, Redmond, WA). A minimum of three biological replicates having no less than four technical replicates were used for each time point.

Recombinant IcmT purification and antibody production. The *C. burnetii icmT* ORF was PCR amplified using CB78 [f] and CB79 [r], and Vent polymerase (New England Biolabs, Ipswich, MA). The resulting PCR product was ligated into the pET200/D-TOPO (Invitrogen) vector, clonally isolated using *E. coli* TOP10 cells, and its sequence verified (clone designation, GA39). The recombinant plasmid (pGA39) was transformed into the *E. coli* BL21 strain DE3 (Invitrogen) and protein expression was induced using IPTG. His-tagged IcmT was purified using nickel chelation chromatography according to the manufacturer's instructions (Thermo Fisher Scientific, Rockford, IL). Recombinant IcmT was used as antigen to produce IcmT specific polyclonal antibody in New Zealand White rabbits following Oklahoma State

University's Institutional Animal Care and Use Committee protocols. Serum was stored at -80°C until use.

C. burnetii IcmT expression analysis. *C. burnetii* NMII infected Vero cells growing on 12 mm glass coverslips in 24 well plates were fixed at 0, 8, 16, 24, 48, 96, and 168 hpi with 4% paraformaldehyde and 0.05% Tween-20 in PBS for 15 minutes at room temperature. Indirect immunofluorescent antibody (IFA) analysis was performed using a guinea pig polyclonal antibody against whole *C. burnetii* NMII and rabbit polyclonal antibody against *C. burnetii* recombinant IcmT. The secondary antibodies were goat anti-guinea pig IgG Alexa Fluor® 555 (red) and goat anti-rabbit IgG Alexa Fluor® 488 (green) (Molecular Probes, Eugene, OR). Micrograph images were captured via a Nikon DS FI1 camera on a Nikon Eclipse TE 2000-S microscope at 400× magnification, with NIS-Elements F 3.00 software. Micrograph capture settings were uniform for all images (Tiff file format). Using a modification of a method previously used in *C. burnetii* studies which employs relative pixel ratios in sample quantitation [264], each micrograph image was analyzed using ImageJ version 1.42n (Wayne Rasband, NIH) software. Five fields of view from each of three biological samples were digitally captured for each time sampled. The matching 555 and 488 images were stacked (paired) and converted to grey scale (8bit, see Figure 4.4 inset). No fewer than five regions of interest (ROI) were then selected from the 555 wavelength grey scale images in a blind fashion. The pixel density within the ROIs from each stacked image were then measured as previously published [265]. The mean pixel densities were then compared to obtain the 555:488 ratio for each micrograph ROI. These individual ratios were then averaged ($\geq$75 individual ratios/time point) to determine the relative expression

of IcmT compared to whole *C. burnetii* NMII. The final 555:488 (*C. burnetii*:IcmT) ratio for each time point was then divided into the 0 hpi ratio to obtain the final IcmT relative expression levels. Statistical significance between each time point was evaluated using single factor ANOVA with a 95% confidence interval with MS Excel 2007 (Microsoft, Redmond, WA).

RESULTS

Transcriptional linkage within the *C. burnetii* T4BSS Region I. The *C. burnetii* T4BSS RI gene linkage map is suggestive of three contiguous ORF groups made up of transcriptionally linked genes (see Figure 4.1). These include: (*i*) *icmX*←CBU1651←*icmW*, (*ii*) *icmV*→*dotA*→CBU1647, and (*iii*) CBU1646←*dotB*←*dotC*←*dotD*←*icmS*←*icmT*. To demonstrate transcriptional linkage between the genes, I performed RT-PCR analysis using oligonucleotide primers (see Table 4.1) designed to span intergenic sequences and/or adjoining ORFs. The diamond-ended lines in Figure 4.1 (a) indicate the position of primers and DNA products that would result from RT-PCR amplification. Using total RNA harvested from Vero cells infected with *C. burnetii* NMII (three weeks pi) as template, amplification products were observed (Figure 4.1 b) for each linkage region: (*i*) *icmW – icmX*, (*ii*) *icmV – dotA*, and (*iii*) *icmT – dotD*, *dotD – dotB*, *dotB* – CBU1646 (Figure 4.1 b). Taken together, these data indicate that the *C. burnetii* T4BSS RI is expressed as three operons without precluding the potential for additional transcriptional regulation within the operons.

De novo synthesis of *C. burnetii* T4BSS genes. It has been previously reported in *Chlamydia trachomatis* and *C. burnetii* that significant amounts of RNA exist within purified preparations of elementary bodies and SCVs, respectively [129, 266, 267]. The

utility of the mRNA carried from one host cell infection to the next is unknown. To determine when *de novo* synthesis of mRNA for *C. burnetii* T4BSS genes begin post infection, I used RT-PCR analysis on total RNA samples collected from *C. burnetii* NMII infected Vero cells, which had been enriched for the *C. burnetii* RNA fraction (J. K. Morgan and E. I. Shaw, submitted). Vero cells were inoculated for two hours with *C. burnetii* NMII (0 hpi represents the end of the two hour inoculation) and enriched total RNA samples were collected at 8 hpi from rifampin treated (+Rif) and mock-treated (-Rif) samples. Using RNA from mock-treated samples as template, RT-PCR produces amplicons representing the presence of full-length mRNA by 8 hpi for *icmT*, *icmV*, and *icmW*; the first genes within each of the T4BSS RI linkage groups (Figure 4.2). In contrast, the rifampin-treated samples show no detectable RT-PCR amplification products for these genes (Figure 4.2). Together, these data indicate that by 8 hpi T4BSS RI transcripts carried into the cell by SCVs had degraded and that *de novo* transcription was occurring for the three genes assayed.

Relative transcript levels of *icmX*, *icmW*, *icmV*, *dotA*, *dotB*, and *icmT* during infection. To determine the temporal expression of mRNA from the three *C. burnetii* T4BSS RI linkage groups over a time course of infection, I used RT-qPCR to quantify the relative amounts of the first and last genes from each RI linkage group. Figure 4.3 shows a graphical representation of the relative abundance for *icmX*, *icmW*, *icmV*, *dotA*, *dotB*, and *icmT* transcripts as a function of time. These data points represent the relative fold ratio as calculated by the $2^{-\Delta\Delta CT}$ method [258, 263] in which I normalized each gene transcript to the *C. burnetii rrs* (16S rRNA), and then to the 96 hpi time point (lowest relative expression level). Based on these data, 0 hpi RNA data (not shown) is

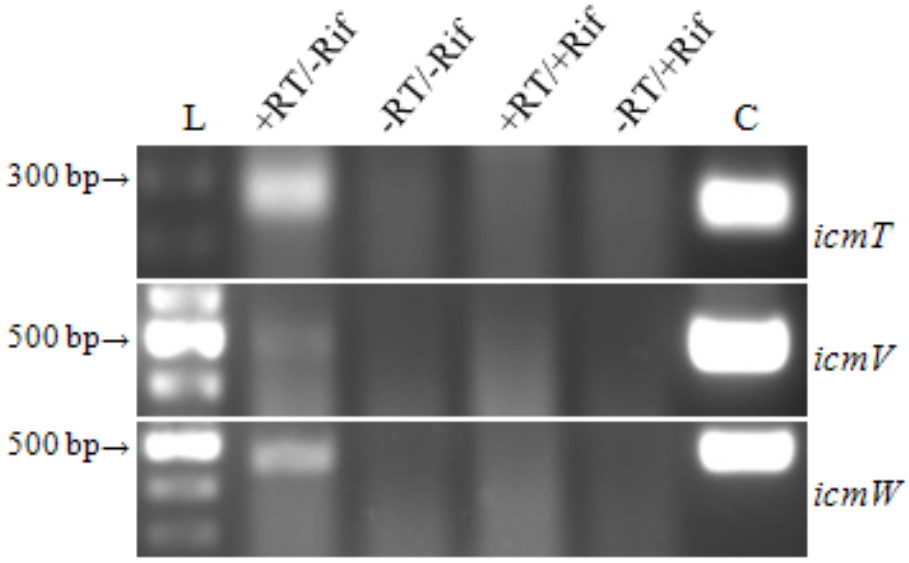

Figure 4.2: RT-PCR detection of full-length *C. burnetii* T4BSS transcripts, *icmT*, *icmV*, and *icmW*. RNA template was isolated at 8 hpi from rifampicin-treated (+Rif) and mock-treated (-Rif) cells. L, 100 bp DNA ladder (size designated on left). +RT/-Rif, with reverse transcriptase and mock-treated. -RT/-Rif, without reverse transcriptase and mock-treated. +RT/+Rif, with reverse transcriptase and rifampin-treated. -RT/+Rif, without reverse transcriptase and rifampin-treated. C, DNA control.

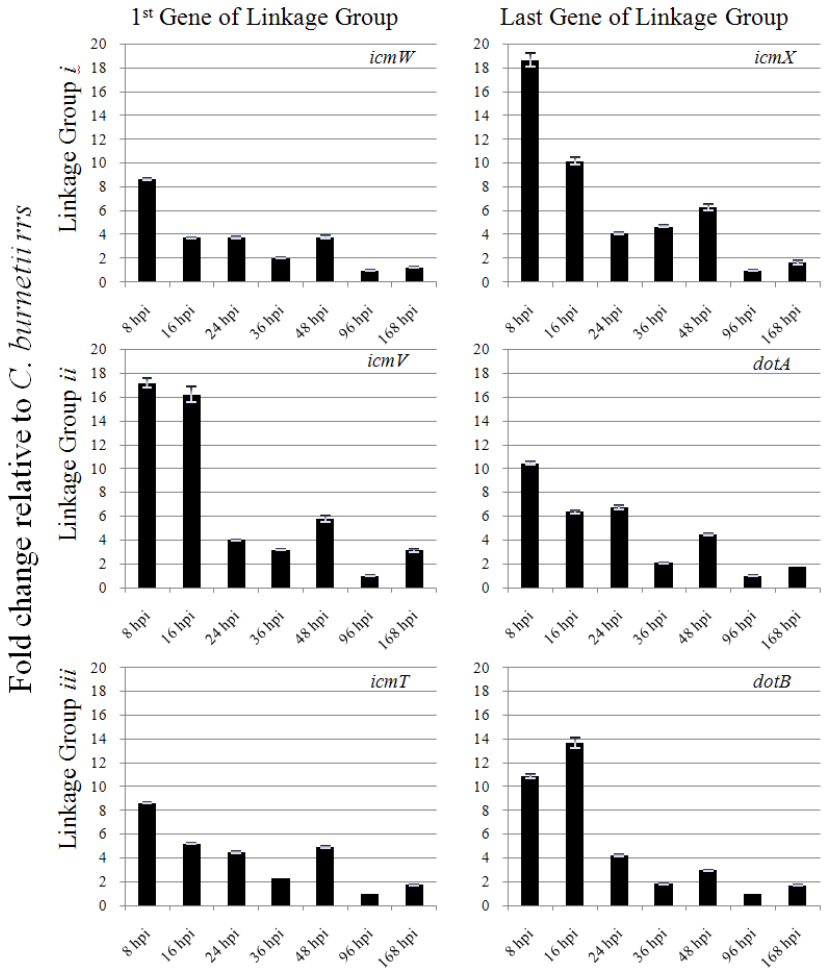

Figure 4.3: Relative transcript fold changes for *C. burnetii icmX*, *icmW*, *icmV*, *dotA*, *dotB*, and *icmT* over a time course of infection. *Left and right columns* are the first and last gene from each Linkage Group, respectively. *Top, middle, and bottom rows* correlate to Linkages Groups *i*, *ii*, and *iii* respectively. Time of total RNA harvest in hpi is indicated below the X-axis. The results represent the mean of three biological samples with no fewer than three technical replicates of each sample. Standard error bars represent the combined standard error of the mean per time point.

attributable to carryover mRNA present within SCVs upon infection, and are not thought to represent functional transcript in the current infection.

Analysis of the 8 – 36 hpi transcript levels indicates that active regulation is ongoing for each of the genes during the early stages of the infectious cycle. This time frame (8 to 36 hpi) corresponds to the late lag phase of a *C. burnetii* infection of a host cell [129]. Analysis of Linkage Group *i* genes indicates that there is a statistically significant decrease in the relative expression of *icmW* between 8 and 16 hpi ($p < 0.05$) and 24 to 36 hpi ($p < 0.05$), and 8 to 24 hpi ($p < 0.05$) for *icmX*. A statistically significant increase ($p < 0.05$) then occurs in the relative amount of *icmX* mRNA from 24 to 36 hpi. Linkage Group *ii* shows a significant ($p < 0.05$) decrease in relative expression from 16 to 36 hpi for *icmV,* and from 8 to 16 and 24 to 36 hpi for *dotA*; however, at 16 and 24 hpi the relative levels of *dotA* mRNA are not statistically different ($p > 0.05$) during infection. In Linkage Group *iii*, *icmT* transcript levels decrease from 8 to 36 hpi ($p < 0.05$); whereas, *dotB* shows a significant increase ($p < 0.05$) in relative mRNA levels from 8 to 16 hpi followed by a significant decrease ($p < 0.05$) from 16 to 36 hpi. Interestingly, transcript levels for each gene analyzed shows a significant increase ($p < 0.05$) between 36 and 48 hpi, then a synchronous decrease in relative transcript levels between 48and 96 hpi, followed by another increase at 168 hpi. These times (96 and 168 hpi) represent the exponential and early stationary phase of the *C. burnetii* infectious cycle, respectively [129].

IcmT expression during infection. To determine the relative expression of a *C. burnetii* T4BSS protein, I analyzed IcmT expression over the course of an infectious cycle. Infected Vero cells were fixed for IFA microscopy at 0, 8, 16, 24, 48, 96, and 168

hpi. Using guinea pig antibodies against whole cell *C. burnetii* NMII and rabbit antibodies against recombinant *C. burnetii* IcmT, I performed dual staining IFA microscopy assays. Figure 4.4, Panels A and B show representative color (RGB) micrograph images illustrating the Alexa Fluor® 555 and 488 secondary antibody binding to the guinea pig (red, Figure 4.4, Panel A) and rabbit antibodies (green, Figure 4.4, Panel B), respectively. Inset panels A and B show 8-bit grayscale micrograph conversions of the RGB images (Figure 4.4, Panels A and B). The pixel data in the grayscale images was used in the analysis of Region(s) of Interest (ROI). The ROI from paired images were equal in area and selected in an arbitrary fashion after conversion of the images from RGB to grayscale. The pixel density of the paired ROIs was used to measure the relative amount of whole *C. burnetii* to IcmT by calculating the 555:488 fluorescence intensity ratios [264, 265]. Cross fluorescent illumination (bleed-through) of both the Alexa 555 and 488 did not occur under these conditions (data not shown). Figure 4.5 is a representation of this data relative to 0 hpi. My analysis revealed that from 0 to 24 hpi, there is a significant increase ($p < 0.05$) in the amount of IcmT relative to whole *C. burnetii* at each time point between 0 to 24 hpi. From 24 to 168 hpi, there is not a statistically significant ($p > 0.05$) change detected and relatively uniform levels of IcmT are maintained throughout the duration of the infectious cycle (Figure 4.5). These data demonstrate that IcmT expression changes during the infectious cycle.

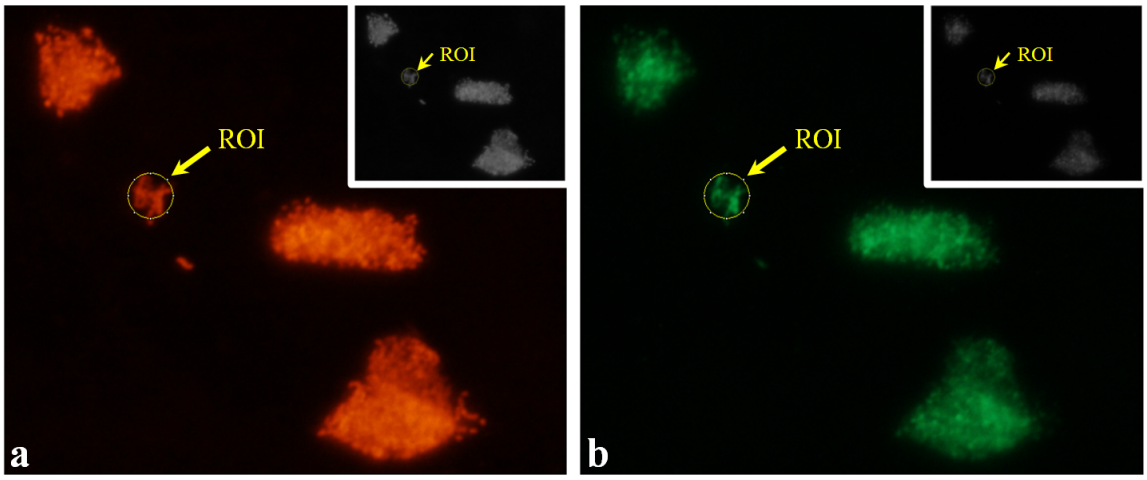

Figure 4.4: Representative dual channel IFA micrograph images (RGB and grayscale) of *C. burnetii* infected Vero cells. Panels a (Alexa® 555/red) and b (Alexa® 488/green), show dual channel micrographs (400X magnification) of the same field of view taken 96 hpi using 555 and 488 wavelength filters. The Alexa® 555 labeled (red) antibody is binding anti-*C. burnetii* guinea pig IgG, and the Alexa® 488 labeled (green) antibody is binding anti-IcmT rabbit derived IgG, respectively. Inset images are 8 bit (gray scale) conversions of the RGB panels. Regions of Interest (ROI).

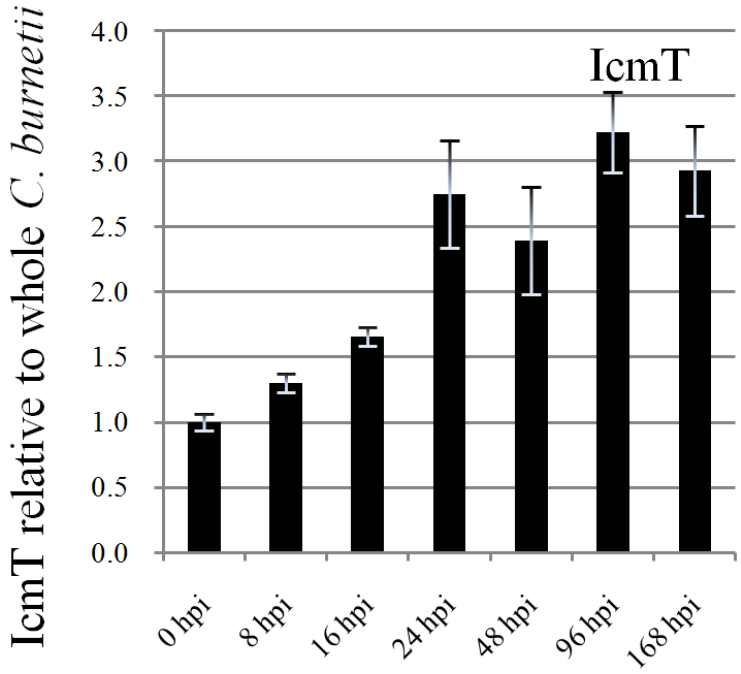

Figure 4.5: Relative IcmT expression over a time course of infection. Pixel density ratios of whole *C. burnteii*:IcmT (555:488) from digital images captured at 0, 8, 16, 24, 48, 96, and 168 hpi were converted to quotient values relative to 0 hpi. Error bars represent the standard error from the mean for each respective quotient value.

DISCUSSION

During a *C. burnetii* infection of a host cell, questions of when the bacteria begin to actively manipulate host cell processes, what bacterial mechanisms are involved, and to what extent this is sustained throughout the infection remain of considerable interest. The homology and similarity of organization between the *C. burnetii* and *L. pneumophila* T4BSS genes [137] suggested that *C. burnetii* possesses a functional T4BSS. To that end, *L. pneumophila* has been utilized as a surrogate model for testing *C. burnetii* T4BSS homolog functionality [213]. Complementation studies using *C. burnetii icmS, icmW, dotB,* and *icmT* homologs in *L. pneumophila* T4BSS mutants demonstrated that these proteins were functional analogs to the *L. pneumophila* proteins. However, *C. burnetii icmQ, dotM, dotL, dotN, dotO,* and *icmX* homologs could not complement the respective *L. pneumophila* mutant [214, 215]. Interestingly, these studies show that four of five *C. burnetii* T4BSS RI genetic homologs were able to complement the *L. pneumophila* T4BSS mutants whereas none of the five RII genetic homologs could complement, suggesting a functional distinction exists between the *L. pneumophila* and *C. burnetii* T4BSSs. Nevertheless, these studies provide strong evidence that the *C. burnetii* T4BSS is involved in host cell parasitism.

Here I sought to determine whether the expression of the *C. burnetii* T4BSS RI genes are temporally regulated during infection by characterizing the transcript levels for *icmX, icmW, icmV, dotA, dotB,* and *icmT* along with the relative protein levels of IcmT. My analysis indicates that the *C. burnetii* RI Linkage Groups (Figure 4.1 a) are expressed as three separate operons (Figure 4.1 b):

(*i*) *icmX*←CBU1651←*icmW*

(*ii*) *icmV*→*dotA*

(*iii*) CBU1646←*dotB*←*dotC*←*dotD*←*icmS*←*icmT*

However, this method does not preclude the possibility for internal gene specific promoters which might further regulate the RI ORFs.

It is well documented that bacterial genes whose products function as a unit are often grouped into operons, and it appears that *C. burnetii* has evolved a similar mechanism for the T4BSS. Sequence data from the *C. burnetii* genome indicate that the T4BSS ORFs within each linkage group have little non-coding intervening sequences [137]. Only *icmW, icmV, icmT, dotD, icmQ,* and *dotP* have more than 90bp of non-coding sequence upstream and none had more than 262 bp. The compact nature of the *C. burnetii* T4BSS contrasts with that of the *L. pneumophila* system in that the *L. pneumophila* T4BSS has non-coding sequences upstream of transcriptional units which range from 91 to 400 bp [217]. Many of the linked *C. burnetii* T4BSS RI genes have very few bases separating them which may allow efficient transcription of the *C. burnetii* T4BSS genes.

Here I show that the initial gene from each of the *C. burnetii* T4BSS RI linkage groups (*icmT*, *icmV*, *icmW*) (Figure 4.1) are synthesized *de novo* as full-length transcripts by 8 hpi (Figure 4.2) using a comparison of rifampin-treated and mock-treated samples. The use of a bacterial RNA synthesis inhibitor to demonstrate *de novo* RNA synthesis provides confirmation that previous studies where *C. burnetii* T4BSS *icmS* and *dotC* transcripts were detected by 8 hpi, *dotB* transcripts by 24 hpi, as well as *icmW* transcripts as early as 6 hpi [249, 250] were likely detecting *de novo* synthesized mRNA. *De novo* synthesis of *C. burnetii* T4BSS *dotA* transcript by 8 hpi was previously implied using

RT-qPCR [129]. Predictably, comparisons of +Rif and mock treated samples harvested later during infection demonstrated that *de novo* synthesis of RNA continued when RT-PCR assays were performed (data not shown). Therefore, it is unlikely that carry-over RNA within an SCV makes a substantial contribution in the translation of proteins during the early stages of infection of a host cell. I used samples harvested at 8, 16, 24, 36, and 48 hpi to analyze the expression of *C. burnetii* T4BSS as it relates to early events of infection such as bacterial trafficking and SCV-to-LCV conversion. While these data show the presence of *C. burnetii* T4BSS transcripts by 8 hpi, it is likely that T4BSS expression may begin even earlier during the infectious process. Electron microscopy evidence showing SCV to LCV conversion by 8 hpi [129] also suggests that bacterial transcription within the host cell may begin earlier during the infection process.

RT-qPCR analysis was used to define the relative expression of the first and last gene of each Linkage Group: (*i*) *icmW* and *icmX*, (*ii*) *icmV* and *dotA*, and (*iii*) *icmT* and *dotB*, over a time course of infection. I used RNA samples harvested at 8, 16, 24, 36, 48, 96, and 168 hpi after synchronous infections were initiated by inoculation with SCVs. Within the *C. burnetii* infectious cycle, these samples correspond to (*i*) early vesicle trafficking and the conversion of SCVs to LCVs (8 to 36 hpi), (*ii*) pronounced metabolic activity and *C. burnetii* LCV replication (48 to 96 hpi), and (*iii*) the beginning of an asynchronous reversion back to the SCV form (168 hpi) [120, 121, 129]. These data showed significant temporal RNA regulation for each of the genes analyzed throughout the time course of infection. In each case, the lowest RNA levels in relation to overall transcription were seen at 96 hpi. All of the genes showed the highest relative expression levels at 8 hpi with the exception of *dotB*, which was highest at 16 hpi. The relative

abundance of transcripts for each of the T4BSS genes analyzed was found to have decreased significantly by 36 hpi (Figure 4.3). Interestingly, each of the *C. burnetii* T4BSS homologs analyzed by RT-qPCR revealed a significant increase in relative transcripts at 48 hpi (Figure 4.3). The 36 to 48 hpi period represents the end of the SCV to LCV lag phase, and the initial burst of replication and a heightened metabolic activity for the bacteria [129]. The trends from 48 to 168 hpi are relatively uniform for all of the genes analyzed with the lowest relative amounts at 96 hpi. However, it is noteworthy that a slight, although significant, increase is detected from 96 to 168 hpi. This corresponds to the asynchronous conversion of *C. burnetii* LCVs to SCVs [129] and may be associated with the mix of LCVs and SCVs present in the PV at that time.

Defining the relative expression of a *C. burnetii* T4BSS protein in relation to its RNA expression during an infection was carried out by measuring the relative quantity of *C. burnetii* IcmT. Using fluorescent microscopy, I observed IcmT 0 hpi indicating it is likely present in infectious SCVs. I observed a significant increase in *C. burnetii* IcmT expression between each time point analyzed during the first 24 hpi (0 to 8 hpi, 8 to 16 hpi, and 16 to 24 hpi, respectively). From 24 to 168 hpi, my observations indicated that the relative IcmT quantity did not change to a statistically significant degree ($p > 0.05$) (Figure 4.5). It is unclear whether the IcmT detected at 0 hpi has functional utility, although two possibilities for the IcmT detected at 0 hpi are: (*i*) it is part of a functional T4BSS structure poised to act upon uptake by a host cell, or (*ii*) it is not part of a functional T4BSS structure but is available for quick assembly once taken up by a host cell. Combined with my RT-qPCR analysis, these data suggest that *C. burnetii* T4BSS protein expression closely follows the increase in *icmT* transcript early during infection (0

to 24 hpi) and becomes relatively uniform for the duration of the infection (24 to 168 hpi). A close comparison of Figure 4.3 (*icmT*) and Figure 4.5 reveals that IcmT expression is higher following a rise in *icmT* transcript levels and decreases following a decrease in transcripts (Figure 4.3 and Figure 4.5, 24 to 48 hpi). High RNA expression early during infection corresponds to a rapid rise in IcmT protein levels from a low at 0 hpi. Predictably, this suggests that transcription leads directly to a subsequent increase or decrease in translation for IcmT. The relationship between the RNA and corresponding IcmT protein expression indicates that temporal regulation of *icmT* transcription and IcmT protein expression exists during the *C. burnetii* infectious cycle.

In summary, I have shown that the *C. burnetii* T4BSS RI is expressed as a set of three operons and that *de novo* transcription and translation of *C. burnetii* T4BSS genes is present as early as 8 hpi. How much earlier expression of these genes may begin is unknown at this time. In addition, I have shown that the first 24 hpi is vital with respect to an increase of both transcription and translation for these genes. Transcript and protein levels for *C. burnetii* T4BSS RI genes at later stages of an infection (48 to 168 hpi) appear to be relatively constant. These data provide a substantial increase in our understanding of the temporal regulation of the *C. burnetii* T4BSS and when this virulence mechanism may be crucial to the bacterium's intracellular life cycle.

CHAPTER V

POLAR LOCALIZATION OF THE *COXIELLA BURNETII* DOT/ICM TYPE IV SECRETION SYSTEM

The zoonotic disease Q fever is caused by *C. burnetii*, an obligate intracellular bacterial pathogen [12] which has only recently been propagated in a cell-free media [251]. During a normal intracellular infectious cycle within its host cell, *C. burnetii* lives in a parasitophorous vacuole (PV) with many of the attributes of a mature phagolysosome [169, 179, 181, 223-226]. Recent studies have shown that *C. burnetii* protein synthesis is required for the pathogen to affect host cell processes, such as apoptosis [229] and vesicle trafficking [187, 188], at sites distant to the PV. Sequence analysis of the *C. burnetii* strain (RSA493) Nine Mile phase I genome revealed a set of genes with homology to the Dot/Icm type IV secretion system (T4BSS) found in *Legionella pneumophila* [137]. In addition, the genome sequence revealed *C. burnetii* ORFs containing eukaryotic Ankyrin binding repeat domains [227, 228]. Subsequently, these ORFs have been shown to be secreted by *L. pneumophila* in a T4BSS dependant manner [227, 228], further implicating the *C. burnetii* T4BSS as a significant factor in cellular pathogenesis. In *L. pneumophila*, the T4BSS system consists of twenty-six ORFs of which twenty-three share significant homology with *C. burnetii* ORFs [137]. Studies have clearly shown that the *L. pneumophila* T4BSS is required for intracellular survival, effector protein secretion, and replication within host cells [202, 203, 244-248]. Thus, the T4BSS plays a vital role in the infectious process of *L. pneumophila*, however characterization of the T4BSS system in *C. burnetii* during an infection is lacking.

Polar localization of bacterial virulence factors has been recognized in both Gram-negative and Gram-positive bacterial pathogens. Type IV secretion systems export virulence factors which includes nucleoprotein complexes and effector proteins that are secreted directly into a host or into the extracellular milieu [194, 196, 199]. The type IV

secretion systems have been subdivided into two families, (*i*) the VirB/D4 (T4ASS) and (*ii*) the Dot/Icm (T4BSS) systems [194]. The T4ASS of *Agrobacterium tumefaciens* has been shown to directly inject effector molecules into adjacent cells [194] as well as into the extracellular environment [233, 234]. VirB8, part of the core complex, was reported to localize at the pole of *A. tumefaciens* cells approximately 50 – 65% of the time [238] and the bacterium has been shown to attach to plant host cells at the bacterial poles [268]. The T4BSS system in *L. pneumophila* is essential for cellular pathogenesis and has been shown to secrete numerous effector proteins into a host cell [199, 231]. In addition, *L. pneumophila* type IVB secreted substrates LidA, SidC, and SdeC, and one T4BSS component, DotF, has been shown to have a polar localization [210, 241-243].

Further virulence factors that have been shown to localize or disperse about the pole(s) of Gram-negative and Gram-positive bacteria include alternate secretion systems, effector protein molecules, and surface membrane associated proteins. For instance, evidence suggests that the type III secretion system of *Shigella flexneri* is present on the poles of the bacteria prior to the secretion of the IpaC protein [269]. Recently, the *Mycobacterium marinum* Esx-1 type VII secretion system was shown to secrete substrate, Mh3864, at the poles and that a core Esx-1 component, Mh3870, localized preferentially to the poles [270]. Type V autotransporter secretion shows polar localizing proteins that are crucial to Gram-negative bacterial virulence and include AIDA-I (*Escherichia coli*), BrkA (*Bordetella pertussis*), IcsA and SepA (*Shigella flexneri*) [271]. The NalP autotransporter from the Gram-negative cocci *Neisseria meningitides* has also been shown to localize to the poles of transformed *E. coli* cells expressing the protein [271]. In addition, the Gram-positive *Listeria monocytogenes* surface protein ActA localizes to

the bacterial pole where it is involved in invasion and actin-based motility [272]. These examples underscore a wide array of mechanisms in which bacterial virulence stratagems have polar localization.

C. burnetii's ability to effect host cell function while sequestered in the PV, and the observation that many secretion systems and virulence mechanisms localize polarly in numerous pathogenic bacteria led me to investigate the sub-cellular localization of the *C. burnetii* T4BSS. Using antibodies specific to the *C. burnetii* IcmT, IcmV, and DotH homologs, I demonstrate by IFA that IcmT, IcmV, and DotH localize to one or both poles of the bacterium and confirm these findings using immunoelectron microscopy. This is the first demonstration of the localization of this virulence machinery during a *C. burnetii* infection of host cells.

MATERIALS AND METHODS

Bacterial Cultivation and Purification. *C. burnetii* Nine Mile Phase II Clone 4 (NMII) was propagated in African green monkey kidney (Vero) cells in RPMI medium with 5% fetal bovine serum (FBS) and the SCV form of the organism was isolated as previously described [129]. The SCV pellet was then resuspended in SPG buffer (0.7M sucrose, 3.7mM KH_2PO_4, 6.0mM K_2HPO_4, 0.15M KCl, 5.0mM glutamic acid, pH 7.4) and stored at -80°C. Organisms were enumerated by genome equivalents using qPCR [138].

Cell culture and infection. Vero cells were propagated in RPMI 1640 media containing 5% FBS with gentamicin (20 µg/ml) at 37°C and 5% CO_2. Culture media without antibiotics was added two hours prior to bacterial infection that was carried out in the same media. Vero cells were infected with *C. burnetii* NMII at an MOI of 0.5,

resulting in 40% infection. Infected cells cultures were incubated in RPMI, 5% FBS at 37°C, and 5% CO_2 for 3 weeks.

Expression plasmid construction. Oligonucleotide primers used for the PCR amplification of *icmT*, *icmV*, and *dotH* from *C. burnetii* NMII genomic DNA were, *icmT*: 5' – *CACC*ATGAAATCTCTCGATGAGG (Forward) and 5' – *TTA*GTTATCCCACCATGCTATGG (Reverse), *icmV*: 5' – *CACC*ATGATTCTTTTGGAGTCTTCC (Forward) and 5' – *TTA*TTGTTTGGACCCCTTAAAGGTG (Reverse), *dotH*: 5' – *CACC*ATGGTGATTCGAAAAATTTTCC (Forward) and 5' – *TTA*CAACCCTTCAATCATCAAC (Reverse). Underlined and italicized bases, *CACC* and *TTA*, are non-*C. burnetii* sequences used for directional cloning and stop codon creation, respectively. Blunt PCR products from each gene were ligated into the pET200/D-TOPO vector and transformed into *E. coli* TOP10 cells according to the manufacturer's instructions (Invitrogen, Carlsbad, CA). Selected clones were cultivated at 37˚C in Luria-Bertani (LB) broth containing 50 µg/ml Kanamycin and sequence verified. The clones were designated GA39 (*icmT*), GA44 (*icmV*), and GA36 (*dotH*).

Recombinant protein purification. Plasmids pGA39, pGA44, and pGA36 were electroporated into *E. coli* BL21 (DE3) cells (Invitrogen, Carlsbad, CA) and protein production was induced following the manufacturer's instructions (Invitrogen). The cells were then pelleted by centrifugation at 6,000 × g for 20 minutes and disrupted in lysis buffer (50mM Tris, 200mM NaCl) using sonication. Inclusion bodies were pelleted at 27,000 × g for 15 min and then solubilized using a modification of a previously described method [273]. Briefly, pellets were washed in lysis buffer with sodium lauroyl

sarcosinate (sarkosyl) at 10% (v/v). The repelleted inclusion bodies were then solubilized using 0.3% sarkosyl in Tris buffer (50mM Tris, 300mM NaCl) and allowed to incubate at room temperature with agitation. Insoluble particulates were cleared by centrifugation at 20,400 × g for 15 minutes. The solubilized His-tagged proteins were purified using nickel chelation chromatography according to the manufacturer's instructions (Thermo Fisher Scientific, Rockford, IL). SDS-PAGE gels and MALDI-TOF or Orbitrap mass spectrometry analysis at the Oklahoma State University Recombinant DNA/Protein Core Facility was used to confirm the content of each recombinant protein preparation. The quantity of purified recombinant proteins was determined using a BCA™ protein assay kit according to the manufacture's specifications (Pierce). Purified recombinant proteins were stored at -80˚C.

Antibody production and purification. The production of polyclonal antibodies against recombinant IcmT, IcmV, and DotH protein was performed in accordance to the Oklahoma State University Institutional Animal Care and Use Committee protocol. Briefly, New Zealand White rabbits were injected with 1mg/ml of recombinant protein in Freund's complete adjuvant (Sigma-Aldrich) for the primary injection and Freund's incomplete adjuvant for subsequent injections. IFA and immune-blotting were used to confirm antibody reactivity and protein specificity. Exsanguination was preformed via cardiac puncture and serum separated from whole blood by centrifugation at 8,200 × g for 15 minutes. IgG fractions were then enriched using Protein-A cross-linked agarose beads according to the manufacturer's instructions (Pierce). Each antibody was then dialyzed against PBS and concentrated using iCON™ spin concentrators (Pierce). The

polyclonal antibodies were then absorbed against *E. coli* BL21 (DE3) cells fixed in a 4% (w/v) paraformaldehyde, 0.05% (v/v) Tween-20 in PBS solution.

Indirect Immunofluorescent Antibody analysis. Vero cells infected with *C. burnetii* NMII (three weeks post infection) were seeded to 12mm glass coverslips in 24 well plates and allowed to adhere overnight. Adherent cells were then fixed using a 4% (w/v) paraformaldehyde, 0.05% (v/v) Tween-20 in PBS solution for 15 minutes at room temperature. Indirect immunofluorescent antibody (IFA) analysis was performed using a guinea pig polyclonal antibody against whole *C. burnetii* NMII and rabbit polyclonal antibody against *C. burnetii* recombinant IcmT, IcmV, and DotH. The secondary antibodies were goat anti-guinea pig IgG Alexa Fluor® 555 (red) and goat anti-rabbit IgG Alexa Fluor® 488 (green) (Molecular Probes, Eugene, OR). To stain bacterial and host Vero cell nucleic acids, 4', 6-diamidino-2-phenylindole (DAPI) was included with the secondary incubation. Micrograph images were captured via a Nikon DS FI1 camera on a Nikon Eclipse TE 2000-S microscope at 600 × magnification, with NIS-Elements F 3.00 software. All micrograph size and merge (RG or RGB) functions were performed universally for the associated micrographs using ImageJ version 1.42n (Wayne Rasband, NIH) software.

Immunoelectron microscopy. To observe the localization of DotH, IcmT, and IcmV at the ultra-structural level, heavily infected Vero cells (see above) were prepared for progressively low temperature embedding IEM. Infected cells were trypsinized, pelleted, and fixed on ice for 1 hour in PBS containing 4% paraformaldehyde (v/v), 0.05% glutaraldehyde (v/v). The Washington University, Department of Molecular Microbiology Center for Infectious Disease Research, Imaging Facility (St. Louis, MO),

performed the sample processing and immune electron microscopy analysis. Samples were embedded in 2% agarose and rinsed extensively in dH_20 prior to en bloc staining with 1% aqueous uranyl acetate (Ted Pella Inc., Redding, CA) for 1 hour. Samples were subsequently dehydrated in a graded series of ethanol at progressively lower temperatures, infiltrated with LR Gold resin (Ted Pella Inc.) at -20°C, and polymerized under UV light. Samples were sectioned with a Leica Ultracut UCT ultramicrotome (Leica Microsystems Inc., Bannockburn, IL). Sections were blocked with 5% fetal bovine serum/5% goat serum for 30 minutes and subsequently incubated overnight at 4°C with primary antibodies against IcmT, DotH, and IcmV, respectively. Sections were then washed in blocking buffer and probed with anti-rabbit IgG (H+L) conjugated to 18 nm colloidal gold (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA) for 1 hour at room temperature. Sections were washed in phosphate buffer followed by an extensive water rinse and stained with uranyl acetate and led citrate. Samples were viewed with a JEOL 1200EX transmission electron microscope (JEOL USA Inc., Peabody, MA). Additional samples were also processed for cryo immunoelectron microscopy as previously described [274]. Cryo IEM sample fixation and section probing were performed as described above for IcmT, DotH, and IcmV. The labeling experiments for both progressively low temperature embedding and cryoimmunoelectron microscopy were conducted in parallel with controls omitting the primary antibody. These controls were consistently negative at the concentration of colloidal gold conjugated secondary antibodies used in these studies.

RESULTS AND DISCUSSION

In an effort to determine the localization of the *C. burnetii* T4BSS, IFA analyses using rabbit antibodies specific to IcmT, IcmV, and DotH was employed. IFA microscopy of Vero cells heavily infected (3 weeks post infection) with *C. burnetii* NMII show bacterial cells with polar and bi-polar localization of the T4BSS proteins when probed with antibodies against *C. burnetii* IcmT (Figure 5.1, panels A and B), IcmV (Figure 5.1, panel C), and DotH (Figure 5.1, panel D) proteins, respectively. Polar localization of the T4BSS proteins can clearly be seen in the enlarged panels (Figure 5.1, panel B and panels C and D insets, arrows). While cell specific localization was difficult to differentiate in densely populated PVs, polarity was readily detectable in spacious PVs (Figure 5.1, panel A) and with individual *C. burnetii* cells (Figure 5.1, panels C and D). Heavily infected cells were used in this analysis in order to capture all aspects of the infectious cycle. While IcmT is detectable at 0 hours post infection (hpi) (defined as the end of a 2 hour inoculation, Morgan et. al, submitted), the polarity is not clearly discernable. However, polarity is clear in IFAs using antibodies to IcmT by 8 hpi (Figure 5.1, panel E) and can be observed throughout the infectious cycle (and data not shown). In addition, I observed bi-polar localization in approximately 60% of the cells where polarity was observed (Figure 5.1, panel B).

To confirm the fluorescent microscopy observations, I analyzed *C. burnetii* infected Vero cells using immunoelectron microscopy (IEM) with *C. burnetii* IcmT, IcmV, and DotH specific antibodies. IEM analyses revealed polar localization of the gold-particle conjugated anti-rabbit antibodies to one and/or both poles of the bacteria when using primary rabbit antibodies to IcmT and DotH (Figure 5.2). IEM results for

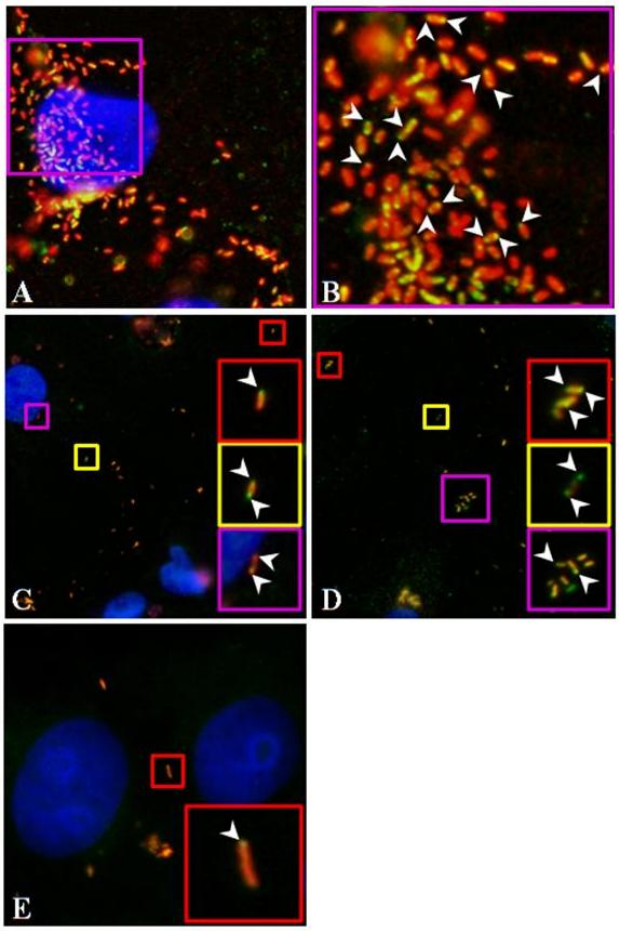

Figure 5.1: IFA localization of *Coxiella burnetii* NMII IcmT, IcmV, and DotH. *Panel A*, Merged IFA micrograph showing whole *C. burnetii* cells in red (goat anti-guinea pig Alexa 555), IcmT in green (goat anti-rabbit Alexa 488), and Vero cell nucleus in blue (DAPI stain). *Panel B*, enlargement of *Panel A*, pink squared area. *Panels C*, merged image detecting IcmV localization, and *Panel D*, merged image detecting DotH localization. *Panel E*, merged IcmT showing polar localization by 8 hours post infection. Enlargements of three cells are shown within inset panels (red, yellow, and pink squares) for each protein.

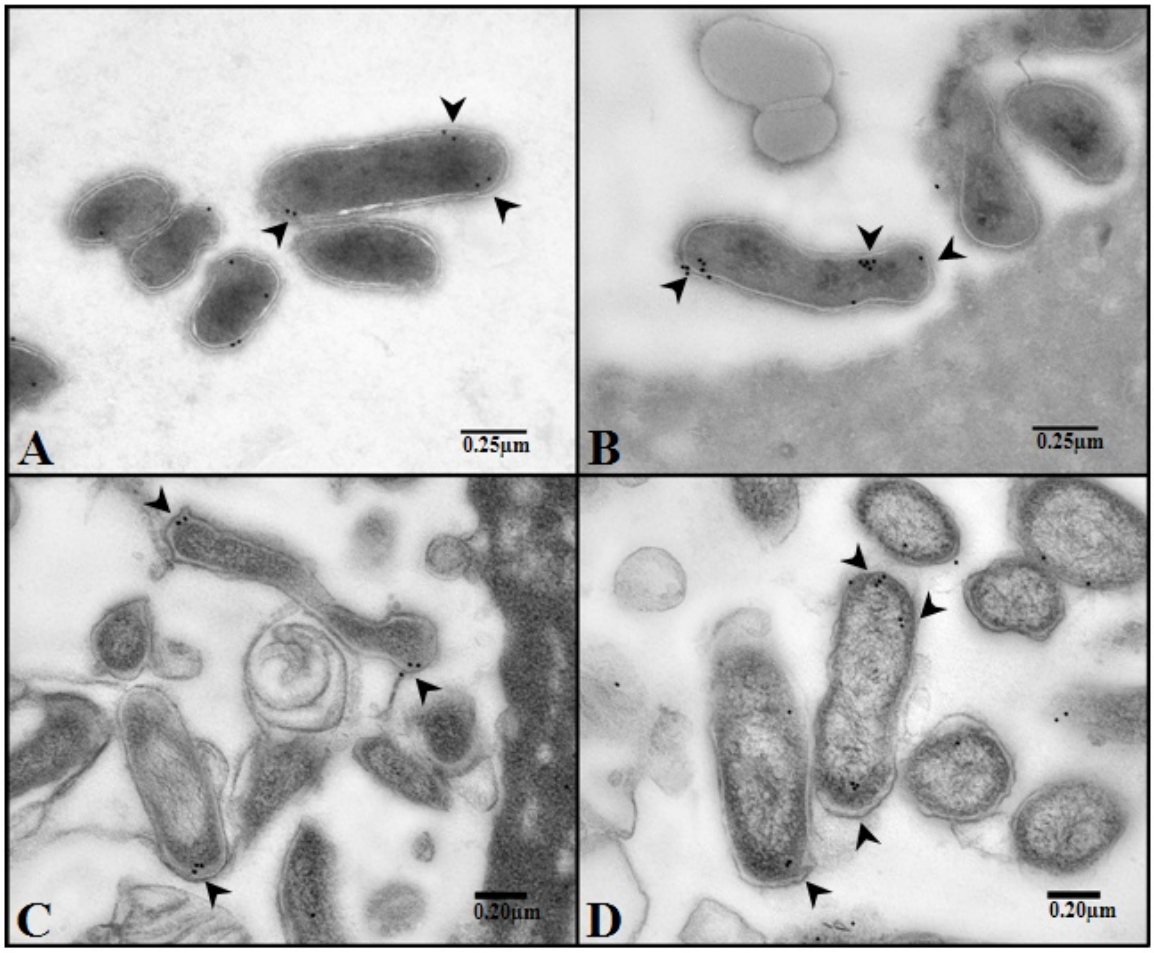

Figure 5.2: IEM localization of *Coxiella burnetii* NMII IcmT and DotH. Panels A and B - Representative cryo-IEM micrographs of *C. burnetii* cells probed with antibodies to IcmT. Panels C and D - Representative low temp-IEM micrographs of *C. burnetii* cells probed with antibodies to DotH. Black arrows indicate immune-gold particles at the ends of *C. burnetii* cells. Size bars and sizes are shown for each image.

IcmT and DotH show both single and bi-polar expression in cells that appear to be LCVs (Figure 5.2), based on an LCV length of 0.5 to 1.0µm [120, 122, 275]. Multiple attempts and technical approaches to obtain conclusive IEM results for IcmV localization were unsuccessful. However, the conclusive results for both IcmT and DotH provided us with evidence of polar localization of the *C. burnetii* T4BSS. These IEM data support the results observed by IFA for both IcmT and DotH localization patterns (Figures. 5.1 and 5.2) as well as IcmV localization observed by fluorescent microscopy.

Using Vero cells heavily infected with *C. burnetii* NMII allowed for ready identification of polarity by IEM however, biological questions remain about the morphology of the T4BSS. For instance, it remains to be determined whether the *C. burnetii* T4BSS initially localizes medial to the polar regions to then migrates lateral to the poles during the course of cellular development, or whether it initially nucleates at the pole(s) and recruits sequential components to the nucleation site. In addition, IFA demonstrating the presence of IcmT by 0 hpi raises the question as to whether SCVs have a functional T4BSS at the outset of infection. It may be that a functional *C. burnetii* T4BSS is carried over from the previous LCV to SCV conversion event or it arises from *de novo* expressed proteins produced very early in infection. It may be that both scenarios may exist simultaneously. It is interesting that I observe bacteria with T4BSSs on one or both poles. The bi-polar localization of the T4BSS on *C. burnetii* cells may correlate to cells that are approaching cell division. This would ensure that progeny cells are each equipped with a functional T4BSS after binary fission. This would imply that bacteria with T4BSS at a single pole are the result of a recent bacterial cell division (Figure 5.1 and 5.2).

The utility of having the T4BSS expressed on the pole(s) of the *C. burnetii* cells and how this may relate to the pathogens interactions with the host cell through the PV membrane is not clear. The observation that *A. tumefaciens* intimately interfaces with its host cell at the bacterial pole [268] and that its T4ASS machinery then secretes effectors into the host [194] would indicate that direct association of a T4SS with a membrane may allow effector secretion across/into the membrane. An analogous interface between pathogen and membrane has been observed in the intra-vacuolar bacteria *Chlamydia trachomatis,* which secretes effector proteins into the host cell via a T3SS [276] and closely associates with the PV (inclusion) membrane during infection [277, 278]. An obvious and consistent interface between a pole of *C. burnetii* and the PV membrane has not been reported. However, a study of published EM micrographs [129, 275] as well as my own (Figure 5.3) indicates that there are instances where the poles of *C. burnetii* contact the PV membrane. Whether these are simply random events, or whether the transient association of the bacterial pole with the PV would allow *C. burnetii* to secrete effector proteins into/through the PV membrane remains to be determined.

In summary, my studies provide the first evidence that the *C. burnetii* T4BSS localizes at one or both poles of the bacterium during infection. The combined IFA and IEM analyses revealed *C. burnetii* with single-pole or bi-polar expression of the T4BSS homologs IcmT, IcmV, and DotH. However, the purpose(s) for polar expression of the *C. burnetii* T4BSS as it relates to the ability of the pathogen to secrete effector proteins into or across the PV remains to be elucidated.

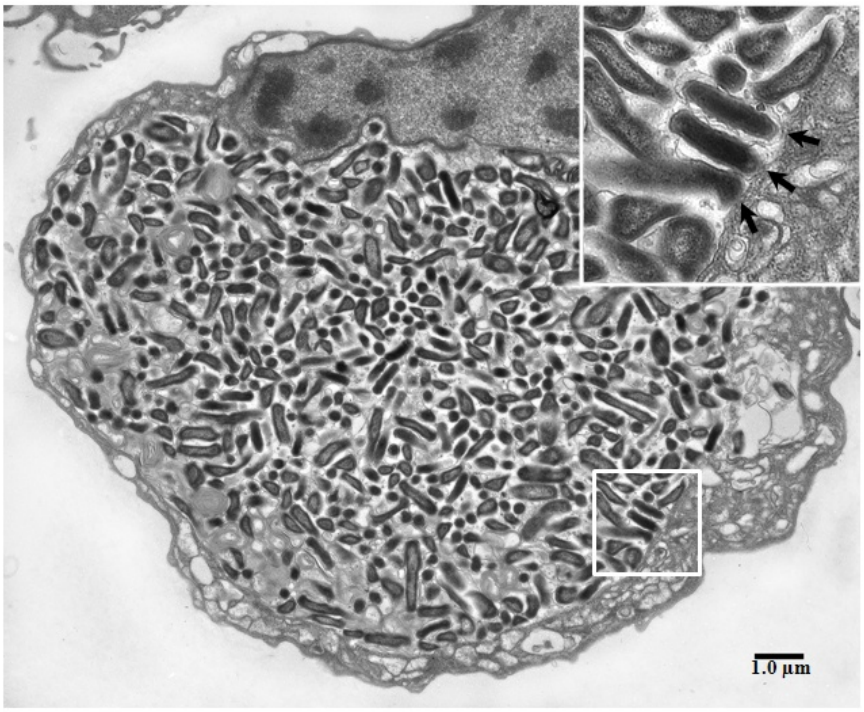

Figure 5.3: Electron micrograph of *Coxiella burnetii* infected Vero cell with densely packed PV. Box Inset: Arrows (black) designate *C. burnetii* cells in polar contact relative to the PV membrane boundary; size bar is 1.0-micron.

CHAPTER VI

SUMMARY REVIEW OF:

ANALYSIS OF THE *COXIELLA BURNETII* TYPE IV SECRETION SYSTEM REGION I DURING INFECTION

Coxiella burnetii is a Gram-negative obligate intracellular bacterial pathogen that is the causative agent of the zoonotic disease Q fever [12]. *C. burnetii* has two main forms. The first is the metabolically inert SCV and the second is the metabolically active large LCV [120]. Though both forms are infectious *in vitro* [122, 128], the SCV is widely recognized as the form implicated in naturally acquired Q fever infections [122]. Typical, naturally acquired Q fever occurs as SCV cells are inhaled into the lungs of the host where it is then endocytosed by alveolar macrophages. Once endocytosed by the host cell, the SCV containing vesicle is trafficked down the endocytic pathway where it becomes acidified by lysosomal fusion, thereby becoming a phagolysosome. This *C. burnetii* containing phagolysosome is known as a parasitophorous vacuole (PV). As the pH of the PV lowers, *C. burnetii* detects this change and begins a metabolic shift from SCV to the LCV developmental form [122].

The sequence of the *C. burnetii* Nine Mile phase I (RSA 493) genome revealed significant homology to the *Legionella pneumophila* Dot/Icm type IV secretion system [137]. The type IV secretion system category is defined by modern equivalents that are ancestrally related to ancient conjugation machinery which have been modified to deliver pathogenic virulence factors (*e.g.*, effector proteins and/or protein/nucleotide complexes) [194, 196, 199]. The type IV secretion category has been divided into two subdivisions, type IVA and IVB [194]. Type IVA systems are homologous to the *Agrobacterium tumefaciens* VirB/D4 secretion system [194, 231, 232], while type IVB secretion systems have homology to the *L. pneumophila* Dot/Icm system [248]. Both type IVA [199, 232, 235] and IVB [202, 203, 244-248] systems have been shown to be essential pathogenic enabling machinery. Genetic mapping of the *C. burnetii* Dot/Icm type IV system genes

has been shown that exist primarily on two loci designated Regions I (RI) and II [137]. Little is known about these regions and the role they play in establishing and/or maintaining *C. burnetii* infections. The *C. burnetii* Dot/Icm type IV RI contains open reading frames (ORFs) that are arranged in three directional groups:

(*i*) *icmX* ←CBU1651←*icmW*

(*ii*) *icmV*→*dotA*→CBU1647

(*iii*) CBU1646←*dotB*←*dotC*←*dotD*←*icmS*←*icmT*

Considering the ORF orientations, I hypothesized that these *C. burnetii* RI ORFs were co-transcribed as operons and that they are temporally regulated both transcriptionally and translationally over the course of infection.

The obligate intracellular lifestyle of *C. burnetii* has limited RNA transcriptional studies to analyses using total RNA harvests. Consequently, the vast majority of the RNA preparations contain predominantly eukaryotic host RNA species. The prevailing eukaryotic host cell RNA found within total RNA harvests significantly limits the analyses that may be performed with respect to *C. burnetii* specific transcriptional studies. Here I demonstrated a modified digitonin lysis technique which combined the stabilizing effects of GeneLock™ (Sierra Molecular) with ice preservation to yield high quality RNA that is significantly enriched ($p < 0.05$) for *C. burnetii* specific RNA relative to conventional total RNA preparations. This method allowed us to perform RT-PCR studies at earlier time points during infection than were previously possible without the enriched *C. burnetii* RNA. Additionally, this technique should provide a means whereby previously impractical *C. burnetii* specific RNA analyses such as microarray and Northern blots may now be practical.

To characterize the transcriptional and translational regulation of the *C. burnetii* Dot/Icm type IV RI ORFs, I analyzed temporal expression profiles of RNA and protein for specific RI genes over a time course of infection. To assess RNA regulation, I performed synchronous *C. burnetii* infections of host Vero cells from which RNA was harvested at early, mid, and late stages of infection, and analyzed these by RT-PCR and RT-qPCR. By RT-PCR, I demonstrated that each of the three RI directional ORF groups were co-transcribed as operons. Additionally, I showed that by 8 hpi, *de novo* RNA transcription was detectable for each of these three operons. Using RT-qPCR, I demonstrated significant ($p < 0.05$) up and down regulation of transcriptional activities within the first 48 hpi for each of the three RI operons. I also showed that relative uniform transcription trends followed from 48 to 168 hpi for each of the three operons. Subsequent to this, I demonstrated by indirect immunofluorescent antibody assay (IFA) the protein expression profile of the RI encoded protein IcmT over the time course of infection. The IcmT protein showed a regulatory trend that tightly correlated with the temporal transcription of the *icmT* gene. Combined, these data demonstrate temporal regulation of both RNA and protein expression within the *C. burnetii* Dot/Icm type IV RI genes, and that these events appear to coincide with *C. burnetii* SCV conversion to LCV and back to SCV.

The findings that the *C. burnetii* T4BSS RI is temporally regulated at both the RNA and protein level, in addition to my discovery that the *C. burnetii* T4BSS is expressed in the bacterial pole(s), provides novel answers to questions concerning *C. burnetii*-host cell interactions while posing additional questions, vis-à-vis *C. burnetii* early temporal regulatory events and T4BSS specific ultra-structural interactions within

the host come. The flurry of transcriptional activity of the *C. burnetii* T4BSS RI genes at early time points (0 to 24 hpi) may well be related to the pathogens need to shape its intravacuolar niche into an environment suitable for its replication before it is digested by the cells normal innate phagolysosomal mechanisms. To discover the physical properties and functions of the *C. burnetii* T4BSS as it relates to these biological events will lead to a more full understanding of *C. burnetii* parasitism specifically and bacterial interactions with host cells in a broader sense.

It has been previously reported that type IV secretion system components and/or substrates appear to localize polarly in bacteria containing either type IVA [236, 237, 239] or IVB [210, 241-243] secretion systems. Using antibodies developed to recombinant *C. burnetii* IcmT, IcmV, and DotH proteins, I investigated the sub-cellular localization of the *C. burnetii* Dot/Icm type IV secretion system by IFA and immunoelectron microscopic (IEM) analyses. Utilizing these methods, I was able to identify direct visual evidence showing *C. burnetii* type IV homologs IcmT, IcmV, and DotH are polarly expressed on *C. burnetii* LCV cells within a Vero host cell background. To my knowledge, this is the first direct evidence for *C. burnetii* Dot/Icm type IV secretion system ultra-structural localization. These data strongly suggest that the *C. burnetii* Dot/Icm type IV secretion system complex localizes polarly in the metabolically active *C. burnetii* LCV cells.

With respect to the finding that the *C. burnetii* T4BSS is localized to the bacterial pole(s), a combined study using IFA and IEM analyses over the time course of infection may provide evidence of protein specific T4BSS ultra-structural membrane localization, individual protein component nucleation, structural assembly, system maintenance, and

host/parasite T4BSS PV during infection. It is not clear at this time whether bacteria with the T4BSS at one pole are the result of bipolar cells that divide, or the result of an independent T4BSS formation event at the pole itself. The possibility also exists that the T4BSS is assemble on the bacterial side and migrates to the as the peptidoglycan layer is made at the midline of the bacteria. The functional advantage of a polarly expressed *C. burnetii* T4BSS is not clear, but may well be related to the pathogens ability to interact with the PV. The possibility of an intimate T4BSS/PV membrane interface may well explain how bacterial proteins secreted within a membraneous vacuole are delivered to the host cell cytoplasm as well as other organelles. Conversely, should a stable interface with the PV not present itself, it may suggest that the effector molecules have the ability to transverse the PV membrane subsequent to T4BSS secretion into the PV lumen by some yet discovered means.

Further study of the SCV and LCV forms of *C. burnetii* by IEM and IFA may well reveal whether the infectious SCV form is prepared to immediately begin secreting effector proteins via the T4BSS upon contact with a eukaryotic cell, or whether the T4BSS is present in non-functioning individual protein components, or whether it is absent altogether and has to be de novo synthesized upon infection. The presence or absence of T4BSS in SCVs will have important implications respecting *C. burnetii* parasitism molecular interactions with the host cell.

CONCLUSIONS

- The combination of digitonin lysis with GeneLock™ performed on ice significantly enriches the relative quantity and quality of intracellular specific bacterial RNA.

- The *Coxiella burnetii* Dot/Icm type IV Region I contains 3 operons that are expressed and coordinately regulated over the time course of infection as the bacteria shifts from SCV to LCV and back to SCV.

- *Coxiella burnetii* Dot/Icm type IV Region I protein expression increases significantly early during infection and correlates to Region I RNA expression.

- The *Coxiella burnetii* Dot/Icm type IV secretion system is polarly expressed on the LCV.

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VITA

John Kent Morgan

Candidate for the Degree of

Doctor of Philosophy

Thesis:

ANALYSIS OF THE *COXIELLA BURNETII* TYPE IV SECRETION SYSTEM REGION I DURING INFECTION

Major Field: Microbiology, Cell and Molecular Biology

Biographical:

Personal Data: Born December 25, 1974 in Salt Lake City, UT USA, the fourth of five children to Dr. Garth R. and Jean P. Morgan. Married Mary A. Morgan August 8, 1998. Three children: Stephen (9 years), Adam (6 years), Emma (4 years), expecting second daughter December 2009.

Education: Graduated from Bonita High School in La Verne, California 1993; Received Associate degree in Liberal Arts from Citrus College in Glendora, California 1997; Received Bachelor of Science from Brigham Young University in Provo, Utah in 2002; completed the requirements for Doctor of Philosophy Degree in Microbiology, Cell and Molecular Biology at Oklahoma State University in Stillwater, Oklahoma in December 2009.

Experience: Team Leader/Content Senior Analyst, InsurQuote (Provo, Utah) January 1998 through April 2001; Laboratory Manager, Microbe Inotech Laboratories, Inc. (St. Louis, Missouri) June 2002 through July 2004; Teaching Associate, Department of Microbiology and Molecular Genetics, Oklahoma State University (Stillwater, Oklahoma) August 2004 through May 2009; Research Associate, Department of Microbiology and Molecular Genetics, Oklahoma State University (Stillwater, Oklahoma) May 2007 through September 2009.

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Name: John Kent Morgan Date of Degree: December, 2009

Institution: Oklahoma State University Location: Stillwater, Oklahoma

Title of Study: ANALYSIS OF THE *COXIELLA BURNETII* TYPE IV SECRETION SYSTEM REGION I DURING INFECTION

Pages in Study: 118 Candidate for the Degree of Doctor of Philosophy

Major Field: Microbiology, Cell and Molecular Biology

Scope and Method of Study: *Scope* - To determine the temporal regulation of *Coxiella burnetii* type IV secretion system Region I homologs during infection of host cells over a time course of infection. *Methods* for microbiological and molecular analyses include: eukaryotic and prokaryotic cell culture, digitonin/GeneLock™ cell lysis, Deoxyribonucleic acid (DNA), Ribonucleic acid (RNA), Polymerase Chain Reaction (PCR), reverse transcriptase PCR, quantitative reverse transcriptase PCR, recombinant protein generation, recombinant polyclonal antibody generation, indirect immunofluorescent antibody (IFA), immunoelectron microscopy, fluorescent and light microscopy, bio-analyzer, etc.

Findings and Conclusions: *Findings* – I found a combination of Digitonin lysis with GeneLock™ performed on ice, significantly enriches the relative quantity and quality of intracellular specific bacterial RNA. I also determined that the *Coxiella burnetii* Dot/Icm type IV RI contains 3 operons that are expressed and coordinately regulated over a time course of infection as the bacteria shifts forms. Which led to my discovery that *Coxiella burnetii* Dot/Icm type IV RI protein expression over a time course of infection shows a tight correlation to RI RNA expression over correlating time points during host cell infection. Additionally I discovered that the *Coxiella burnetii* Dot/Icm type IV secretion system is polarly expressed on the LCV bacterial form. *Conclusions* – I conclude that the *C. burnetii* type IV secretion system region I is temporally regulated at both the RNA and protein level within its host over its time course of infection correlating to phase shift from SCV→LCV→SCV bacterial forms and that the *C. burnetii* type IV system is expressed on the bacterial poles in the LCV form.

ADVISER'S APPROVAL: Edward I. Shaw, Ph.D.